A study of wheat endosperm development: cell and starch granule numbers and amyloplast DNA and RNA

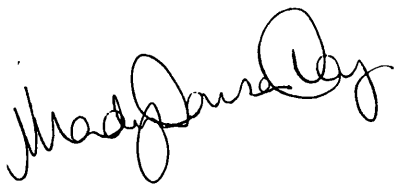
Day, Mandy Dowson

Award date:
1987

Awarding institution:
University of Bath

A STUDY OF WHEAT ENDOSPERM DEVELOPMENT:
Cell and Starch Granule Numbers
and
Amyloplast DNA and RNA

submitted by Mandy Dowson Day
for the degree of Ph.D.
of the University of Bath, 1987



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I would like to thank Janet Pryke for her encouragement and optimism througout, and with Mike Bayliss, Wolf Schuch, Keith Edwards (at I.C.I. Jealott's Hill and Runcorn) and Cathy Bowman (at the P.B.I., Cambridge) for invaluable advice and instruction. I would also like to thank Beryl Scutt for typing the thesis so well and Colin Wilson (at the Photographic Department, Bath University) for the excellent A4 photographs.

Many thanks also to Chris for all his support and to our families for all sorts of things.

I am grateful for the receipt of a C.A.S.E. award from the S.E.R.C., in conjunction with I.C.I. plc.

SUMMARY

Sterilisation of the basal florets of Triticum aestivum (cv Timmo) resulted, after 40 days of growth until maturity, in a mean fresh and dry weight increase of the third floret grains of 43.8% and 40.0%, respectively; and a 53.4% increase in endosperm cell number. The rate, but not duration, of DNA synthesis and cell division increased, although the mean cell ploidy was not significantly affected. Mean endosperm cell dry weight was reduced by up to 19.6%.

Grain removal caused the total number of endosperm starch granules and the number of A type granules to increase by 33.7 and 28.3%, respectively. The final A type modal volume was not significantly affected, although starch granule and A type amyloplast number per cell fell by up to 19.2% and 17.6%, respectively. It is suggested that the increase in grain size represents the unexploited capacity of the grain and a model for the control of grain size is proposed.

The presence of nucleoids in A type amyloplasts, as visualised by DAPI-staining, is discussed. Southern blots revealed the likely existence of ptDNA in total wheat endosperm DNA and that this hybridises strongly to wheat ctDNA. The percentage ptDNA as a proportion of total in leaves (11.1%) and endosperms was quantified by dot blots probed with the ctDNA fragments P6 and B2: in endosperms this increased from approximately 0.74% to 0.91% during grain filling and then fell to 0.57% at the onset of maturation. The number of plastome copies per endosperm, per cell and in relation to the cell nDNA content were determined.

RNA dot blots revealed a low level of transcripts with homology to P6 in total endosperm RNA throughout development. Southern blots of ctDNA fragments spanning the plastome were probed with total leaf and endosperm RNA. The latter could be detected hybridising to only the ribosomal DNA fragments P6 and S8.

ABBREVIATIONS

ABA	: abscisic acid
NSC	: non-structural carbohydrate
d.p.a.	: days post anthesis
ptDNA	: plastid DNA
O.R.F.	: open reading frame
ctDNA	: chloroplast DNA
TCA	: trichloroacetic acid
PCA	: perchloric acid
endo-S	: endoreduplication phase
endo-G	: non-DNA synthetic phase
Sarcosyl	: N-lauroyl sarcosine
Tris	: 2-amino-2-(hydroxymethyl)propane-1,3-diol
EDTA	: ethylenediaminetetraacetic acid
ethidium bromide	: 3,8,-diamidine-5-ethyl-6-phenyl-phenanthridium bromide
DNase	: deoxyribonuclease
BSA	: bovine serum albumin
ccctDNA	: closed circular chloroplast DNA
nDNA	: nuclear DNA
DAPI	: 4';6-diamidino-2-phenylindole dihydrochloride
MES	: 2-[N-morpholino)ethane sulfonic acid
DTT	: dithiothreitol
spermine	: (N,N'-bis[3-aminopropyl]-1,4-butandiamine
DABA	: diaminobenzoic acid
TTP	: thymidine 5'-triphosphate
dATP	: deoxyadenosine 5'-triphosphate

dCTP	: deoxycytidine 5'-triphosphate
dGTP	: deoxyguanosine 5'-triphosphate
PPO	: 2,5 diphenyloxazole
mtDNA	: mitochondrial DNA
rRNA	: ribosomal RNA
ctRNA	: chloroplast RNA
RNase	: ribonuclease
SDS	: sodium dodecyl sulphate
PVP	: polyvinylpyrrolidone

CONTENTS

CHAPTER 1

INTRODUCTION

The worldwide importance of Triticum aestivum and other cereal grains cannot be overestimated. They provide two thirds or more of the dietary calories in the less developed countries and more than half of the energy consumed in a number of developed countries (Evans and Wardlaw, 1976). In 1978, wheat was the largest world food crop yielding more than 400 million tonnes, followed closely by Oryza sativa and Zea mays. Despite the economic importance of these cereals, factors controlling the number of grains per spike and the ultimate size of a grain are not well understood; even the pathway whereby starch is synthesized in non-photosynthetic storage tissue has not yet been conclusively determined (Boyer, 1985; Salerno, 1986; MacDonald and ap Rees, 1983; Gross and ap Rees, 1986; MacDonald and Preiss, 1986).

The need to improve the production efficiency of both starch and protein is critical, given the restrictions on further increases in land area under crops (Evans and Wardlaw, 1976). Breeding programmes and improved agronomic practise have produced an increase in wheat yield, in England, from approximately 0.4 tonnes per hectare in the mid thirteenth century to more than 5.3 tonnes per hectare in the late 1970's (Evans, 1981); an increase of at least two fold being achieved over the last twenty five years (Riley, 1981). However, it is being increasingly recognized that a more

detailed knowledge of the physiology, biochemistry and genetics of cereal grains is necessary if these improvements are to continue. Evans (1981) suggests that although it is possible to simply select empirically for increased yield, an alternative approach, offering the potential for more radical advance, would be to identify first the physiological processes limiting grain yield. He suggests that the plant breeder could then select for specific improvements. Assumably such data should also encourage the plant molecular biologist to determine the feasibility of manipulating the plant genome with the same aim in mind.

Since starch represents from 70 to 80 percent of the dry weight of a mature wheat grain (Jenner, 1982a) it is important to extend our understanding of both the biochemistry and control of starch deposition. This project was initiated to contribute to the latter through the study of the pattern of starch granule synthesis in large and small wheat grains of the same genotype and by investigating the potential role of wheat endosperm plastid DNA in the starch-containing organelles.

1.1 The wheat grain and endosperm

The tissues in the mature wheat grain originate from three distinct genomes. The outer layers, comprising the nucellar tissue, integuments and chalazal tissues (Fig. 1.1), have their origins in the diploid, maternal ovule walls and stalk: these degenerate to form the seed coat, 'testa', and the pigment strand. Within these lies the pericarp, cross

cells and tube cells, which are also maternal tissue, derived from the ovary walls (Simmonds and O'Brien, 1981). These layers encompass and provide assimilate to the non-photosynthetic endosperm and embryo tissues which arise from two separate fertilisation events. The triploid endosperm originates from the central cell of the embryo sac, containing two haploid, polar nuclei, and one of two haploid, male nuclei from one pollen grain (3n = 9x = 63). The second male nucleus fuses with the haploid nucleus of the egg cell to form the zygote and eventually the embryo (2n = 6x = 42) (Bennett et al., 1973).

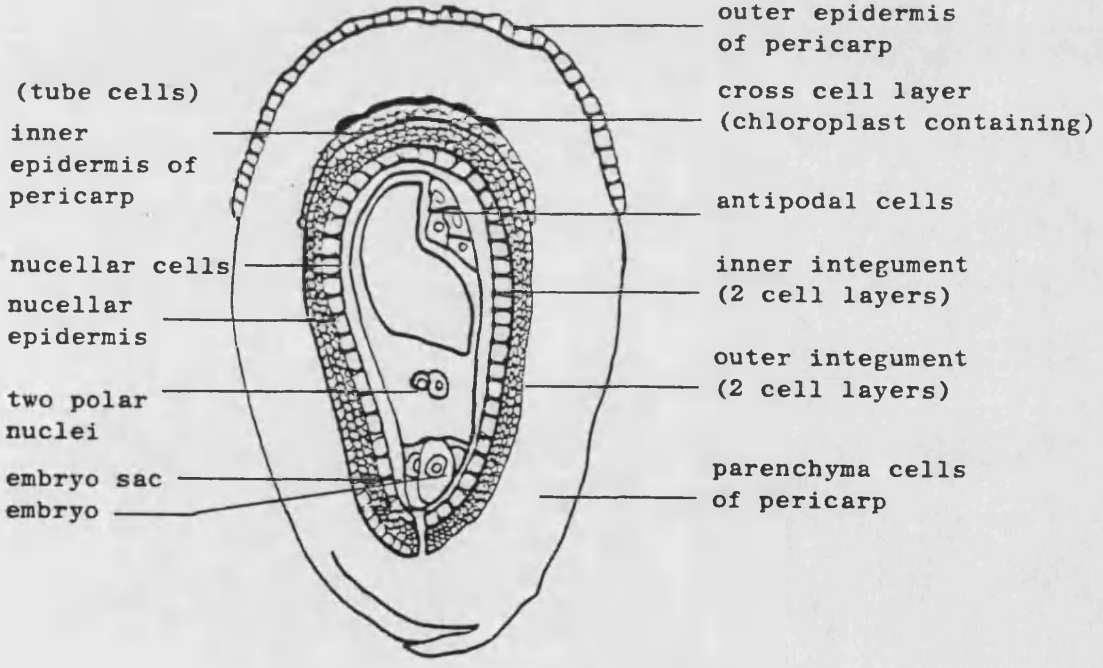


Fig. 1.1 Diagram of the wheat ovule prior to fertilization showing maternal cell layers and embryo sac (based on Baldo et al., 1982).

Evers (1974) described endosperm development as occurring in five phases, the first being the formation of the first triploid nucleus. The second encompasses the period of synchronous free nuclear division and is followed by cellularisation, cell division and cell enlargement, the third phase. The fourth is characterised by the differentiation of an outer layer of cells, which are meristematic and contain little starch, the 'aleurone'. During this phase most of the storage reserves of the grain are accumulated and considerable cell enlargement occurs. The final phase encompasses the process whereby the grain, which has attained maximum fresh and dry weights, loses water to become ripe, 'maturation'.

Since the endosperm contains two sets of maternal chromosomes and only one set from the paternal plant it is not surprising that the maternal genes, for the gliadin storage proteins, have been found to be expressed at twice the level of those from the paternal plant (Simmonds and O'Brien, 1981 and references therein). It would appear that this bias towards the maternal genotype is likely to be of significance in a consideration of genes controlling the synthetic potential of the endosperm.

The zygote undergoes up to ten cycles of mitotic division without the formation of cell walls (Bennett et al., 1973); with mean cycle times of 4.7 h for the first five division cycles and 9.6 h for divisions six to ten. Wall ingrowths produced from the edge of the embryo sac branch and join so that the endosperm becomes cellularised (Morrison et

al., 1978; Bechtel et al., 1982). After this the cycle time of cell division extends to approximately 16 h and is no longer synchronous (Bennett et al., 1973). The initial cells are highly vacuolated with only a parietal layer of cytoplasm; those at the perifery divide by a regular pattern of radial, 'anticlinal', and tangential, 'periclinal', divisions (Morrison et al., 1975). Each dividing cell gives rise to a radial file of cells, the oldest being furthest removed from the meristematic outer layer. Later divisions result in denser, less vacuolated cells which tend to be rich in storage protein reserves. Cells that were initiated earlier are characterised by their large size and high starch content (Simmonds and O'Brien, 1981; Evers, 1971). Changes in cell number and volume and the numbers and volume fractions occupied by various organelles have been estimated by stereological analysis of the developing endosperm (Briarty et al., 1979).

Endosperm starch is deposited as granules which fall into two major classes (Sandstedt, 1946; Baruch et al., 1979). The first to be initiated are the A type granules, which at grain maturity range from 8 μm to 45 μm or more in diameter and become lenticular in shape (Evers, 1971; 1973). Their maximum size, of from 35-50 μm diameter, depends on the cultivar (Evers, 1971; Simmonds and O'Brien, 1981; Sandstedt, 1946) and environmental conditions (Baruch et al., 1979; Brooks et al., 1982). The B type granules, which are initiated later, remain spherical or polygonal in shape and range from 1 to 10 μm in diameter (May and Buttrose, 1959

Evers, 1973; Buttrose, 1963). In addition there may be a minor component of underdeveloped A types from sub-aleurone cells (Baruch et al., 1982).

Single A type granules are enclosed within the double membranes of the endosperm amyloplasts (Parker, 1985). By definition the amyloplast is a starch-containing plastid which primarily functions as a storage compartment or may, in roots, be involved in gravitropism (Kirk and Tilney-Bassett, 1978; Jenner, 1982a). It is considered probable that the amyloplasts are derived from proplastids associated with the polar nuclei but it is not known whether organelles accompanying the male pollen nucleus may also contribute a proportion of the endosperm population, although this appears unlikely (Day and Ellis, 1984). As cellularisation proceeds the immature amyloplasts increase rapidly in number and size, concurrently developing internal tubules formed by the invagination of the inner membrane (Morrison and O'Brien, 1976). The tubules become oriented about a groove around the equatorial region of the developing granule (Evers, 1971). It is considered likely that these tubules are involved in starch deposition (Buttrose, 1960, 1963). Electron micrographs have revealed that the B type granules are initiated within the stroma of A type amyloplasts, often within narrow protusions extending into the cytoplasm (Buttrose, 1963; Parker, 1985). These granules, which are less closely associated with the amyloplast tubules, do not have an equatorial groove characteristic of the A types (Buttrose, 1960). It is not clear whether B type granules

ever separate from the A type amyloplasts prior to maturation nor whether their starch composition differs from that of the A types (Parker, 1985).

1.2 Grain yield potential: an evolutionary perspective

Evans (1981) describes yield potential as the 'yield of a cultivar grown in environments to which it is adapted, with nutrients and water non-limiting and with pests, diseases, weeds, lodging and other stresses effectively controlled.' There are a number of genetic components which, although they significantly affect grain yield, are not considered to be determining components of the yield potential. These include adaptedness to agriculture (non-shattering ears, non-dormant grains), adaptedness to different environments (timing of the life cycle), survival in adverse conditions (heat, drought and cold), resistance to pests and diseases and to fertilizers and agrichemicals (short stemmed wheats). Further consideration of these is beyond the scope of this thesis which will be limited to those factors understood to determine wheat yield potential and will discuss components of this list only where they contribute to this understanding.

In the course of wheat evolution there has been an increase in the number of grains per spikelet as well as a substantial increase in grain size (Evans and Dunstone, 1970; Austin *et al*., 1980). In contrast, the number of spikelets per ear has changed little. In parallel with increases in grain size, leaf size has also increased, from the wild

diploids to the cultivated hexaploids (Jellings and Leech, 1984). However, these improvements have not been reflected in rice and other crops, the ploidies of which have not changed (Evans, 1981). Increased grain size has been a product of both more rapid and longer grain growth (Sofield et al., 1977a) and this has been accompanied by an increase in the cross-sectional area of phloem in the culm (Evans et al., 1970). In addition, a greater proportion of the photosynthate is translocated from the leaves to the grains in modern wheats, thus the proportion of dry matter invested in the harvested grain, the 'harvest index', has increased. The extended duration of grain growth can, at least in part, be attributed to the fact that the flag leaves of the modern cultivars retain their photosynthetic capacity for much longer periods (Evans, 1981).

It is interesting to note that the growth rate of seedlings has not increased (Evans and Dunstone, 1970; Evans, 1981) nor has leaf photosynthetic rate (Gifford and Evans, 1981). In fact the latter has tended to fall, the highest rates occurring in the wild diploid T. boeticum, which has a very high rate for a C3 plant (Evans and Dunstone, 1970). Whether this decline in photosynthetic rate in the hexaploid wheats is due to a decreased mesophyll cell surface to volume ratio (Dunstone and Evans, 1974; Migniniac-Maslow et al., 1979) or to intrinsic differences in the Hill reaction rates (Zelenskii et al., 1978) is not clear.

Although a number of the modern varieties are slightly faster growing (Austin et al., 1980; Davidson and Birch,

1980), this does not necessarily confer a long term advantage since faster vegetative growth may result in lower grain weight per ear (Warrington et al., 1977; Rawson, 1970). As a result the total crop dry weight, for a given input, has changed little, the most significant varietal increase in yield being due to improvements in harvest index.

1.3 Grain yield potential: determining factors

The three broad determinants of grain yield potential are (i) tiller number, (ii) grain number per spike and (iii) grain weight.

(i) Tiller number

Tiller number per unit area up to approximately 1000 ears per square metre is closely related to grain yield (Evans, 1981) and is determined not only by sowing density and agronomic inputs but also by variety and irradiance (Stockman et al., 1983). Wheat canopies with a leaf area index of four to six are able to intercept almost all photosynthetically active radiation, thus stands with leaf densities of more than this would not be expected to result in increased yield, rather they may be associated with a greater incidence of disease (Evans, 1981). Despite these limitations it is considered that by modifying leaf dimensions and inclination it may be possible to attain greater yields in denser stands (Donald, 1981).

(ii) Grain number per spike

In situations where tiller number per unit area is low, the number of grains per spike may increase, within the limits of the harvest index, and thereby compensate for a possible reduction in yield potential. Grain number is a product of the success of several stages of ear development: spikelet and floret initiation and growth, degree of fertilisation success and the number of fertilised florets aborted. For grain number to increase the ear has to be able to compete more effectively with other developing sinks for the available assimilate supply. Prior to anthesis the ear is primarily competing with the flag leaf (Rawson, 1970), the other, upper leaves and the stem (Fischer and Stockman, 1980), because the leaves are rapidly expanding and the stem is laying down carbohydrate reserves. It was found that when shading treatments were imposed on the young ear the stem received a disproportionately large amount of the reduced assimilate supply (Fischer and Stockman, 1980). Yield potential may also be reduced by the production of late tillers which is likely to adversely affect both grain number and size (Evans, 1981).

In addition to shading (Stockman et al., 1983) and low illumination (Mohapatra et al., 1983b) both water stress (Saini and Aspinall, 1982; Morgan and King, 1984) and high temperature (Saini et al., 1983; Mohapatra et al., 1983b) have been found to reduce the number of fertile florets; the effects being most deleterious around the time of pollen meiosis (Saini et al., 1983; Zeng et al., 1985). It has been

suggested that high temperatures may affect floret development not by decreasing available sucrose but *via* a different process (Mohapatra *et al.*, 1983b; Zeng *et al.*, 1985). High temperatures were found to reduce the rate of initiation of spikelet primordia which resulted in a decrease in the number of spikelets produced (Mohapatra *et al.*, 1983b). Short photoperiods, which delay the onset of spikelet initiation, were also found to prolong its duration with a resultant increase in spikelet number, within genotypically determined limits (Pinthus and Nerson, 1984).

Since infertility has been induced by abscisic acid (ABA) treatments (Zeng *et al.*, 1985) and endogenous levels of ABA have been correlated with varietal differences in fertility and grain set (Morgan and King, 1984; Zeng and King, 1986), it has been considered that ABA may be a common mediator regulating fertilisation success (Zeng *et al.*, 1985). However, the role and mode of action of ABA *in vivo* are not clear: the weight of current data indicates that ABA and the availability of assimilate are not interrelated (Saini and Aspinall, 1982; Waters *et al.*, 1984; Zeng *et al.*, 1985; Munns *et al.*, 1979; Aspinall, 1984). Despite the uncertainty as regards ABA it is evident that any factor environmental or varietal, causing a reduction in assimilate supply to the young ear, particularly at the reproductive stage, is likely to effect a reduction in the number of grains set (Evans, 1981; Fischer and Stockman, 1980).

In addition, any factor which restricts the ability of the ear to compete for assimilate is likely to have a similar

effect. Hendrix et al. (1986) found that for four wheat cultivars the accumulation of non-structural carbohydrate (NSC) in the inflorescences up to seven days prior to anthesis, and their subsequent utilisation, was related to the number of competent florets. Previous reports had indicated a positive correlation between the level of water-soluble carbohydrates in inflorescences immediately prior to anthesis and the number of grains set (Fischer and Stockman, 1980; Stockman et al., 1983; Waters et al., 1984). Hendrix et al. (1986) also presented further evidence that the developing stems and inflorescences compete for assimilate since there was a significant correlation between grain number and the ratio of fructans in the inflorescences and stems. They suggest that the ability of the young ear to convert translocated sucrose to immobile fructans probably helps to establish it as a strong sink.

(iii) Grain size

Grain size is determined by cell number and cell size, which result from the rate and duration of both cell division and of the synthesis of storage materials, respectively. Just as tiller number may be compensated for, by changes in grain number per spike, so grain number may be compensated for by changes in grain size (Sofield et al., 1977a; Pinthus and Millet, 1978; Thorne, 1973). For this reason it has been suggested that yield improvements are most likely to be achieved by developing the potential size of the grain, since there are no subsequent determinants of yield which can

compensate (Chojecki, 1985). Once the grains have started to develop, growth of other sinks, including roots and shoots, has largely ceased, although stem weight may continue to increase in some varieties (Wardlaw, 1970) and under optimal conditions (Thomas et al., 1979). The grains therefore become the dominant sink for assimilate and reserves: the rate at which ^{14}C-labelled assimilate passes into the ear has been found to be directly related to the number of developing grains (Wardlaw and Moncur, 1976).

Although it is not clear how assimilate is translocated to and into the grain and endosperm (Cook and Evans, 1983; Jenner, 1985a,b,c), there is considerable data concerning how the position of a grain within the ear determines its ability to compete for assimilate. Cook and Evans (1983) conclude that the relative size and distance of competing sinks from the source of assimilate largely determines assimilate partitioning, although there is a bias in favour of movement up the ear and along the spikelets. That these effects are due to vascular connections is probable since a spikelet on the opposite side of the ear from the source receives a far smaller share of assimilate when competing with a spikelet on the same side as the source (Cook and Evans, 1983).

The vascular system within the spikelet also bears some relation to the ability of the different floret positions to develop a grain (Hanif and Langer, 1972). The first three florets in a spikelet have direct connections to the rachis, sharing the vascular system of the rachilla (see Fig. 2.2), whereas more distal florets were found to be supplied by

'sub-vascular' elements, which originate from the base of the previous floret (Zee and O'Brien, 1971; Hanif and Langer, 1972). It is the lower florets, in particular the two basal florets, which are nearest the rachis, that are most likely to set grain. However, there is not an invariable relationship between vascular supply and grain development since the first spikelets in the awn contain smaller and fewer grains than those around the middle, even though they all appear to have a similar vascular system (Hanif and Langer, 1972; Cook and Evans, 1983).

The number of cells in the endosperm has been shown to be positively related to grain size both within and between different wheat varieties (Brocklehurst, 1977; Gleadow et al., 1982; Radley, 1978; Singh and Jenner, 1982a, 1984). Cell number is an heritable trait (Gleadow et al., 1982; Chojecki et al., 1986a; Cochrane and Duffus, 1983) which may be limited by the prevailing environmental conditions (Nicolas et al., 1984, 1985; Singh and Jenner, 1984; Jenner, 1979, 1980), probably by a reduction in the supply of assimilate (Singh and Jenner, 1984; Brocklehurst, 1977). It appears that the basal florets in each spikelet are less likely to be restricted, as regards cell number, than the more distal florets which receive proportionately less sucrose under drought stress (Nicolas et al., 1985).

It was found that the rate of cell division is more likely to be restricted than the duration, under limiting conditions (Wardlaw, 1970; Singh and Jenner, 1984). For this reason, the period of rapid cell division, usually up to 15

days post anthesis (d.p.a.), shows the greatest magnitude of response to imposed treatments or environmental stress (Brocklehurst, 1977; Asana et al., 1969). It is not known how a reduced assimilate supply effects a reduction in cell number since it has been found that the absolute concentrations of sucrose, other soluble sugars and amino acid concentrations in the endosperm are not likely to be limiting the rate of cell division (Singh and Jenner, 1982b, 1984). It has been suggested that substances produced by more dominant grains may regulate cell number at other positions (Singh and Jenner, 1982b; Evans et al., 1972; Rawson and Evans, 1971).

Cell size, like cell number, is a product of both environmental and heritable factors, which determine the rate and duration of starch and protein synthesis. Dry matter deposition is often described in terms of a source-sink relationship (Thorne, 1973), where the assimilate supply, 'source' (Fischer and HilleRisLambers, 1978; Nicolas et al., 1984; Brooks et al., 1982) and the intrinsic ability of the endosperm to take up and incorporate the available assimilate, 'sink', (Jenner, 1982a; Jenner and Rathjen, 1972a,b; 1978; Gleadow et al., 1982) are compared with respect to their importance. Jenner and Rathjen (1972a,b) argue that since the influx of sucrose into grains is saturated at relatively low external levels of sucrose, the assimilate supply is unlikely to be limiting starch deposition in all but the most extreme conditions. It was suggested that it may be the passage of sucrose into the

grain, for example phloem unloading (Jenner 1985a,b,c), which determines, in part, the rate of starch synthesis in different varieties (Jenner, 1973, 1974, 1976). It was found that, for a particular wheat variety, the rate of starch synthesis is proportional to the endosperm sucrose concentration but that the concentration necessary to maintain a particular rate was not constant between varieties (Jenner and Rathjen, 1978). From this data it was concluded that there may be genetic variation in the kinetics of the conversion of sucrose to starch or of the transfer of intermediates within the cell.

Varietal differences in the duration of grain filling have also been reported (Sofield et al., 1977a), but it is considered that these account for less of the variation in final grain weight than differences in the rate (Herzog and Stamp, 1983; Gleadow et al., 1982). High temperatures (Donovan et al., 1983a; Bhullar and Jenner, 1985, 1986a) and drought (Nicolas et al., 1984) have both been found to limit the duration of grain filling. High temperatures can also cause the rate of dry matter deposition to increase which, under less extreme temperatures, may compensate for the shortened duration (Wardlaw et al., 1980; Donovan et al., 1983a). Nicholas et al. (1984, 1985) suggest that the duration of grain filling is possibly more sensitive to adverse conditions than is the rate but that this also is unlikely to be due to a decrease in the available assimilate. Under both drought and high temperature conditions they found that the endosperm sucrose concentration remained constant

for all bar the most distal grains of distal florets.

The cessation of grain filling accompanies the decline in photosynthetic activity of both the flag leaf (Gleadow et al., 1982) and the pericarp (Duffus, 1979). However, it is suggested that ultimate grain size is not determined by the termination of an adequate assimilate supply but by a decreased synthetic capacity of the cells (Jenner and Rathjen, 1975, 1977) or by a decline in activity of the maternal grain tissue (Felker et al., 1983). It may be that the maternal pericarp physically restricts cell expansion, as is thought to be the case in pea seeds (Davies, 1976); but the degraining experiments of Radley (1978) which resulted in larger but less dense grains, with enlarged endosperm cavities, indicate that the control may be exerted closer to or within the endosperm.

On maturation, the grain rapidly dehydrates and the glumes and pericarp lose their chlorophyll content. It has been suggested that grain water loss is a result of increased pericarp permeability (Radley, 1976) or, alternatively, that it is due to a reduced water supply following the deposition of lipids in the chalazal zone between the vascular tissue and endosperm (Cochrane, 1983; Zee and O'Brien, 1970; Sofield et al., 1977b; Nicolas et al., 1984). It has been found that the level of endogenous ABA peaks prior to the reduction in water content (McWha, 1975; Radley, 1978) and that exogenously applied ABA hastens the loss of chlorophyll in the glumes (Radley, 1978). If pericarp photosynthesis plays an important role in supplying oxygen to the endosperm

(Duffus, 1979) the cessation of this activity may cause the endosperm to become anoxic, thereby inhibiting dry matter synthesis and grain expansion (Gifford and Bremner, 1981).

In summary, it appears that floret initiation and grain set are tightly limited by assimilate supply and the well being of the plant from approximately ten days prior to and five days after anthesis (Evans, 1978). Once grain number has been determined it is common for assimilate supply to cease to be a limiting factor since other, previously competing, sinks decline in their importance. Hence it is usually intrinsic factors within the grain, possibly involving phloem unloading and starch synthesis (Kumar and Singh, 1980) which subsequently restrict the rate of grain development. With these controls only as much seed is set as the plant is likely to be able to support to maturity under the prevailing conditions. Thus the plant is unlikely to partition assimilate between large numbers of less viable seed (Harper et al., 1970).

1.4 A model system for investigating grain size

Grain removal experiments where half of the spike was excised (Nösberger and Thorne, 1965; Radley and Thorne, 1981) or the majority of spikelets have been removed (Jenner, 1980; Cook and Evans, 1983) or the basal grains in each spikelet have been removed (Radley and Thorne, 1981; Radley, 1978) have been used to investigate the mechanisms controlling grain filling. The surviving grains usually grow considerably larger than their counterparts in intact ears

(Fischer and HilleRisLambers, 1978; Jenner, 1979; Radley, 1978). Radley and Thorne (1981) found that removal of the two basal grains at 6 d.p.a. resulted in the formation of grains in the third floret with an increased dry weight, water content and reducing sugar, amino acid and total nitrogen content.

There is currently little data revealing how grain size is controlled and how this control is affected by degraining, although it appears unlikely that the increase is caused solely by the increased availability of sucrose (Jenner, 1980). Despite this, an understanding of how grains of a particular phenotype attain a larger size when this unknown control is altered is relevant for future manipulation of grain yield potential. It was the intention of this project to investigate further the physiological changes in the endosperm which contribute to the larger size of these grains, in particular to determine the endosperm cell number (chapter 2), starch granule number and size distribution (chapter 3) and cell DNA content (chapters 2 and 4).

1.5 The role of plastid DNA in endosperm amyloplasts

Plastid DNA (ptDNA) in chloroplasts is known to be transcriptionally active, coding for at least 73 genes (Shinozaki *et al.*, 1986) and as many as 28 unidentified open reading frames (O.R.F.'s) (Ohyama *et al.*, 1986) covering a large proportion of the 120-180 kb genome (Rochaix, 1984; Palmer, 1985). DNA homologous to chloroplast DNA (ctDNA), within a particular species, has been found in a variety of

non-photosynthetic plastids, including chromoplasts (Hansmann et al., 1985; Herrmann, 1972; Lobov and Petrov, 1982; Iwatsuki et al., 1985), etioplasts (Cannon et al., 1985a), leucoplasts (Carde, 1984) and amyloplasts (Macherel et al., 1985,1986b; Bondar et al., 1979; Aguettaz et al., 1987; Steele Scott et al., 1984). However in these other plastids the ptDNA has often been found to be present in lower copy numbers (Aguettaz et al., 1987; Cannon et al., 1985a) and to be considerably less, if not at all transcriptionally active (Aguettaz et al., 1987; Macherel et al., 1986b; Piechulla et al., 1986).

The primary objective of this project, in addition to investigating starch granule distribution as a component of grain yield potential, was to investigate whether wheat endosperm amyloplast DNA is present and maintained throughout grain filling and, if so, whether the levels are independent of the cell nuclear DNA content and rates of plastid division (chapter 4) (Butterfass, 1973,1983; Olszewska et al., 1983; Ellis and Leech, 1985).

One of the current indications from studies of factors determining wheat yield potential is that the uptake of sucrose and its conversion to starch is, under most conditions, likely to be a limiting factor (Jenner and Rathjen, 1978; MacDowell Dale and Housley, 1986; Rijven, 1986). As a consequence it is essential that not only the biochemistry of starch synthesis in the endosperm (Kumar and Singh, 1980) but also the molecular genetics of the regulatory enzymes (Chourey et al., 1986; Springer et al.,

1986) and inhibiting factors (Macherel et al., 1986a) should be better understood. All of the genes studied so far have been found to be nuclear encoded or do not appear to be maternally inherited (Chourey, 1982; Kurzok and Feierabend, 1986; Reeves et al., 1986). It is therefore probable that those enzymes having a stromal location are translated on cytoplasmic ribosomes, as preproteins, probably with a plastid or amyloplast specific amino terminal transit peptide sequence (Karlin-Neumann and Tobin, 1986) and transported across the amyloplast envelope, before being cleaved to yield the mature protein (Smith and Ellis, 1979; Robinson and Ellis, 1985). Any enzymes located within the amyloplast tubules presumably have a different presequence (Smeekens et al., 1986; Chua and Schmidt, 1979; Hageman et al., 1986; Colman and Robinson, 1986).

From current data it appears unlikely that wheat amyloplast DNA plays a major role encoding enzymes involved in starch biosynthesis nor for plastid DNA replication or plastid division. However, it may be important for the maintenance of other amyloplast functions such as translocator activities (Höinghaus and Feierabend, 1985), the import of specific proteins or the synthesis of tubule proteins. It was therefore also the intention of this project to investigate whether the amyloplast DNA is transcribed and, if so, to determine which regions of the plastid genome are most active (chapter 5).

CHAPTER 2

WHEAT ENDOSPERM DEVELOPMENT: CELL NUMBER AND PLOIDY OF LARGE AND SMALL GRAINS OF THE SAME GENOTYPE

2.1 Introduction

Grain and spikelet removal experiments have shown that wheat grain size in both basal (Pinthus and Millet, 1978; Bremner and Rawson, 1978) and distal (Radley, 1978; Radley and Thorne, 1981; Bremner and Rawson, 1978) floret positions may increase considerably over the size of comparable grains from intact ears. From these experiments it is apparent that grain size is restricted by a limiting or inhibitory factor, under normal growth conditions. As already discussed (chapter 1), it is unlikely that this can be attributed solely to limitations in the availability of assimilate (Jenner, 1980), therefore another explanation has been sought which may involve some kind of hormonal control (Pinthus and Millett, 1978; Rawson and Evans, 1970; Radley, 1976,1978, 1981). If this supposition proves to be correct it may be possible to increase grain size by, in some way, removing this restriction.

The extra grain capacity or potential attained in degraining experiments has been considered as part of the 'grain yield potential', as described by Evans (1981) (Bremner and Rawson, 1978). However, because under normal growth conditions it is not fulfilled, it will clarify the

discussion here if it is considered as the unexploited capacity of the grain.

The purpose of the work described in this chapter was to determine the magnitude of this unexploited capacity for third floret (C) grains (Fig. 2.1), from a Spring wheat variety (cv. Timmo). It was intended that if, after degraining, a large increase in grain size was apparent, this variety would serve as an easily handled, model system for determining what constitutes this extra capacity. Of particular importance was the characterisation of the DNA contents, cell numbers and ploidy distributions of the two grains, large and small.

Grains from the first, most basal floret position (A) were also sampled. This was in order to investigate the early stages of grain development from cell division through to the period of most rapid starch deposition and to relate the data obtained to the levels of plastid DNA (chapter 4) and RNA (chapter 5) in the endosperm.

2.2 Materials and Methods

2.2.1 Growth conditions

Spring wheat (Triticum aestivum, L. cv Timmo) seeds were surface sterilized in 5% sodium hypochlorite, for 10-15 min, rinsed in sterile water and incubated in the dark on moist filter paper for 48 h at 4°C. The temperature was then increased to 25°C until germination when the seeds were sown in John Innes no. 1 compost. After 4 weeks seedlings were potted in John Innes no. 3 compost, one or two plants in each 15 cm diameter pot, and transferred to either a growth chamber or a greenhouse.

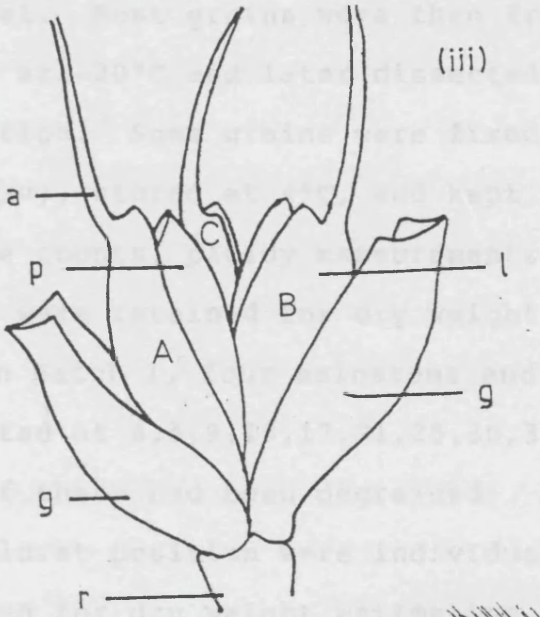
Batches 1, 2 and 4 were grown in a Saxcil growth cabinet (R.K. Saxton, Ltd.) with 16 h, 15°C light and 8 h, 10°C night temperatures. Light was provided by 28 'warm white', 65/80 watt fluorescent tubes and two 40 watt tungsten bulbs. Batches 3.1 and 3.2 were grown in a greenhouse, from February to April, with temperature fluctuations of 3-37°C and 13-40°C, respectively. Supplementary light was provided by 'solarcolour', 400 watt bulbs (GEC) for 16 h each day. Relative humidity ranged from 77% to 95% in the growth cabinet and to as low as 44% in the greenhouse (Hygrometric Tables, 1964).

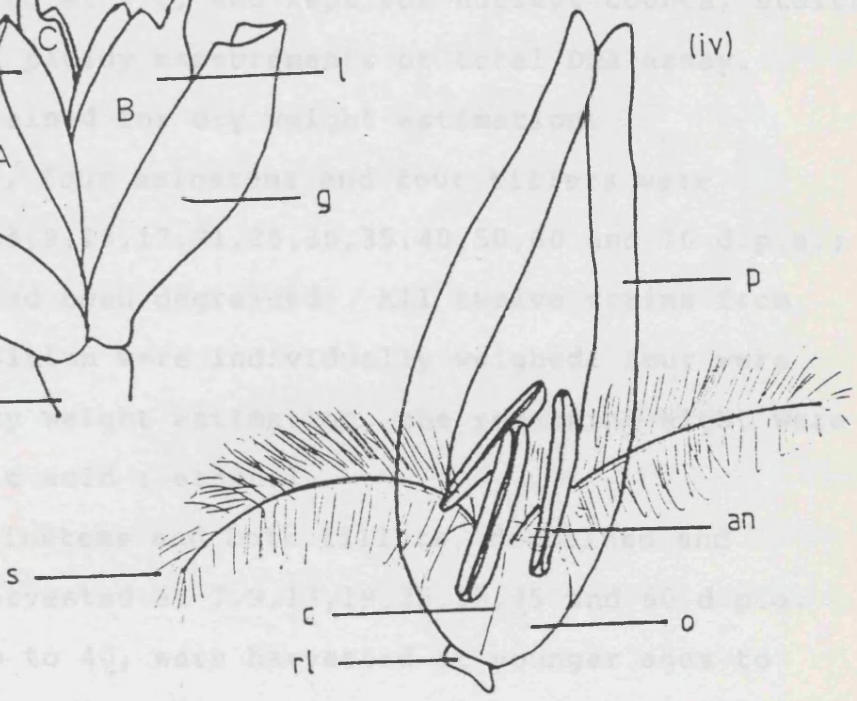
When flowering stems began to elongate tillers were removed leaving three stems per plant (Fig. 2.2(i)). On the appearance of a single anther, anthesis date was recorded, the spike tagged and spikelet number reduced leaving twelve central spikelets per spike (Fig. 2.2(ii)). For Batches 1 and 2, half of the anthesing spikes were degrained.

Figure 2.2 Wheat (Triticum aestivum, cv Timmo) (i) Two plants, each with one mainstem and two tillers. (ii) An ear at anthesis prior to reduction of spikelet number to twelve. (iii) Diagram of a spikelet showing position of basal florets A and B and distal floret C: r = rachis, g = glume, l = lemma, a = awn and p = palea. (iv) Diagram of a floret, with lemma removed: rl = rachilla, o = ovule, c = crease, an = anther and s = stigma.


(i)


(ii)


(iii)
a
p
C
B
A
l
g
g
r


(iv)
p
an
s
c
o
rl

Degraining involved removing the A and B ovules from the basal florets of each spikelet using watchmakers' forceps. The bracts of the A and B florets and the intact C and D florets were left to develop (Fig. 2.2(iii) and (iv))(Radley and Thorne, 1981).

Occasional powdery mildew (Erysiphe graminis) infections were treated with Milgo E and an aphid infection of greenhouse grown plants was treated with pyrethrum (both from I.C.I plc).

2.2.2 Harvesting procedure and methods of storage

Spikes were harvested at various ages after anthesis and the grains segregated depending on their position in the spikelet. Most grains were then frozen in liquid nitrogen, stored at -20°C and later dissected for nucleic acid extraction. Some grains were fixed in acetic acid : ethanol (1:3 v/v), stored at 4°C, and kept for nucleus counts, starch granule counts, ploidy measurements or total DNA assay. Others were retained for dry weight estimation.

In Batch 1, four mainstems and four tillers were harvested at 4,5,9,13,17,21,25,30,35,40,50,60 and 70 d.p.a.; half of these had been degrained. All twelve grains from each floret position were individually weighed: four were retained for dry weight estimation, the remaining eight were stored in acetic acid : ethanol.

Batch 2 mainstems and both tillers, degrained and intact, were harvested at 7,9,13,19,25,35,45 and 60 d.p.a. More spikes, up to 40, were harvested at younger ages to

provide sufficient endosperms for DNA extraction. Grains from each floret position for each spike were pooled, weighed and frozen.

Batch 3.1 mainstems and first tillers from 200-300 plants were harvested at 5,9,13,17,21 and 26 d.p.a. The spikes were immediately frozen and the grains were weighed later prior to nucleic acid extraction. Seven grains, from different spikes, for each age and floret position were used for dry weight estimation. Batch 3.2 grains from at least 20 representative spikes were weighed, sorted into weight categories and stored in acetic acid : ethanol. Seven grains from each group were retained for a dry weight curve. Weight categories chosen were: 0-5, 5-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70 and 70-80 mg fresh weight.

Batch 4 mainstems and tillers from 100 plants were harvested at $7\frac{1}{2}$-8, $8\frac{1}{2}$-9, $9\frac{1}{2}$-10, 12, 14, 17 and 20 d.p.a. Grains were weighed and frozen in weight categories ready for nucleic acid extraction (weight categories chosen were based on the mean weight for each age). Six grains from each category were kept for dry weight estimation.

2.2.3 Measurement of grain fresh and dry weights

Grains from each floret position were weighed, as described in section 2.2.1, before being oven dried at 60°C for 4-8 weeks, by which time grain weight was constant. The difference between corresponding fresh and dry weight means was taken as the grain water content (Nicolas *et al.*, 1985).

2.2.4 Endosperm preparation

Grains stored in acetic acid : ethanol were rehydrated through a series of ethanol concentrations: 70%, 50%, 30% to distilled water, each for 45 min to 1 h, prior to being dissected. Grains stored frozen were dissected as soon as they began to thaw and were immediately refrozen.

A binocular microscope (magnification x 6-15), two pairs of watchmakers' forceps and a small volume of water were used for dissections. Endosperms were prepared by peeling away all the layers of the pericarp and stripping out the vascular and nucellar material in the crease. More mature grains were difficult to dissect, as the pericarp became thinner and tighter, so the vascular material was removed first (Singh and Jenner, 1982b; Simmonds and O'Brien, 1981). The embryo was discarded at all stages of grain development.

2.2.5 Measurement of endosperm DNA content

Perchloric acid extraction of nucleic acid

Grains from Batches 1 and 3.2 were used to determine the total DNA content of C and A grain endosperms, respectively. Nucleic acids were extracted from the tissue using a modification of Smillie and Krotkov's (1960) selective phosphate extraction procedure.

Grains which had been stored in acetic acid : ethanol were transferred to and stored in methanol at 4°C; to remove methanol soluble phosphate. They were rehydrated through a series of decreasing methanol concentrations, the endosperms excised and embryo tissue discarded, as described in section

2.2.4. Replicate samples were used, each containing two or three endosperms.

Acid soluble phosphates were extracted by 2 x 1 ml of 10% trichloroacetic acid (TCA)(AR Grade). Lipid soluble phosphates were extracted by 2 x 1 ml of 95% ethanol, at 2°C, for 2 x 10 min; then 2 x 1 ml of ether for 2 x 10 min. Nucleic acids were extracted from the endosperms into 3 x 0.8 ml of 5% perchloric acid (PCA) (AR Grade) at 70°C for 3 x 45 min. Each PCA extraction was centrifuged at 13,000 x g in a MSE MicroCentaur, the supernatant retained and the pellet resuspended in PCA. The supernatants were pooled and made to 3 ml with 5% PCA.

The efficiency of this DNA extraction procedure was first verified. (i) Increasing the number of TCA washes from two to three or four did not have a significant effect on estimates of DNA content for replicate grains at 25, 40 and 70 d.p.a. ($P \leq 0.05$): this was checked to ensure that DNA was not lost at this stage. (ii) DNA extracted from fresh endosperms gave results comparable to those for endosperms which had been stored in acetic acid : ethanol for two years. (iii) The efficiency of the extraction of DNA using PCA was determined for grains at 25, 40 and 70 d.p.a.: the first and second extractions yielded means of 83% and 17%, respectively, of the detectable DNA; DNA was detected in neither the third wash nor the final pellet.

Diphenylamine assay procedure

DNA was assayed by the reaction of deoxyribose with

diphenylamine (Burton, 1956; Giles and Myers, 1965). All work was carried out in a fume hood. Diphenylamine solution was prepared immediately before use: 4% w/v diphenylamine (Sigma Chem. Co.) was dissolved in Aristar glacial acetic acid (BDH Chemicals Ltd.) then 1% v/v 18M H_2SO_4 (A.R. Grade) was added and the solution kept in an airtight container. DNA dissolved in 5% PCA was mixed 1:1 v/v with diphenylamine solution, then 5% v/v 1.6 mg/ml acetaldehyde was added. The mixture was incubated at 30°C, overnight, in capped, silicon coated glass tubes (see section 3.2). Absorbance of the blue product was measured at 595 nm and any background absorbance, due to turbidity, was measured at 700 nm and subtracted (Chojecki et al., 1986a):

absorbance of sample =

$$(A_{595} \text{ sample} - A_{700} \text{ sample}) - (A_{595} \text{ blank} - A_{700} \text{ blank})$$

Standard curves were prepared using duplicated dilutions up to 100 µg/ml salmon testes or calf thymus DNA (sodium salts, Types I and III, Sigma) in 5% PCA. DNA concentrations of standard solutions were verified by measuring 260 and 280 nm absorbances, using a nomograph to indicate purity, as described in section 3.2.5.

2.2.6 Preparation of Feulgen Reagent

The Feulgen stain, which complexes with the aldehyde groups of DNA and RNA, was used for both direct nucleus counts (section 2.2.7) and nuclear ploidy measurements by

fluorescence (section 2.2.8).

Feulgen reagent was prepared by a modification of the Stowell procedure (1945)(Cosgrove Keown et al., 1977), using acid washed glassware: 200 ml of distilled water was boiled and 1 g of basic fuchsin (pararosaniline hydrochloride (224) CI42500, Eastman) added slowly, to avoid excess frothing. (The crystals were handled wearing gloves in a fume hood.) The mixture was cooled to 50°C, filtered through two Whatman no. 1 filter papers and 30 ml of 1M HCl and 3 g of potassium metabisulphate were added. The solution was left in the dark for 24 h before adding 0.5 g of Noirt, activated charcoal, shaking thoroughly and refiltering. The resultant, colourless Feulgen reagent was stored at 4°C in a light-tight container.

2.2.7 Endosperm nucleus counts

To estimate nuclei number per endosperm, nuclei were Feulgen stained as described by Rijven and Wardlaw (1965) and counted (Singh and Jenner, 1982). This technique was employed for only a few ages of grains from Batch 1 plants, both intact and degrained. Grains aged 20, 25, 30 and 35 d.p.a., stored in acetic acid : ethanol, were rehydrated and the endosperms excised as described in section 2.2.4. There were an average of four replicate grains in each category.

Each endosperm was chopped into 1-2 mm slices, incubated in 1 ml of 4M HCl for 30 min at room temperature, to allow penetration of acid, and pelleted at 13,000 x g for 15 s. The pellet was resuspended in 1 ml of 4M HCl at 40°C and hydrolysed for 15 min, then the centrifugation repeated and

the pellet resuspended in 1 ml of Feulgen (section 2.2.6) and incubated for 2 h at room temperature.

Excess stain was removed by pelleting and resuspending the tissue in 3 x 1 ml of SO_2 water : 2.5 g of $K_2SO_2O_5$ and 25 ml of 1M HCl, diluted to 500 ml with distilled water. Each wash was for 10 min, the sample being centrifuged for 5 min at 13,000 x g. The tissue was buffered by being pelleted and resuspended in 3 x 1 ml of McIlvaine's Citrate-phosphate buffer (pH 5.0): 0.1M citric acid and 0.2M Na_2HPO_4 in the ratio 24.3:25.7 (Gomori, 1955). The pellet was then resuspended and digested in 1 ml of filter-sterilized 1% Macerozyme and 1% cellulase (Onozuka, R-10; both from Yakult Biochemicals Co. Ltd., Japan) in citrate-phosphate buffer at 37°C for 30 min. The nuclei were pelleted as before, washed in 1 ml of 25% acetic acid, repelleted and finally diluted to 2 ml with 25% acetic acid.

Volumes from 50-100 μl were diluted to 20 ml in water and the whole volume filtered, under vacuum, onto a 1.2 μm pore size Millipore filter (Millipore Corporation, U.S.A.). The filters were mounted in glycerol and the stained nuclei counted using a light microscope with an eyepiece graticule.

2.2.8 Measurement of endosperm ploidy

Feulgen staining and slide preparation

The Feulgen stain procedure adopted was largely as described by McLeish and Sunderland (1961). Grains used were C grains, from intact and degrained spikes (from Batch 1) and A grains (from Batch 3.2). All grains had been fixed in

acetic acid : ethanol and then transferred to methanol. They were rehydrated through a series of decreasing methanol concentrations (section 2.2.4), rinsed in 3 x 1 ml of water, for 3 x 20 min, and the endosperms excised. Duplicate endosperms were processed and three slides produced for each. Endosperms were chopped into 2 mm slices to ensure reasonably comparable staining of younger and older grains.

Experiments were carried out to optimise the hydrolysis treatment so that the stained tissue showed intact, highly fluorescent nuclei with acceptably low levels of background fluorescence. Details of these experiments are presented in Appendix I. The most reproducible results were obtained by hydrolysing the tissue in 1M HCl at 60°C for between 2.5 to 7.5 min: for data produced here, tissue was hydrolysed in 1 ml of 1M HCl at 60°C for 6 min. (Microfuge tubes and acid were preheated to 60°C.) The acid was then removed, the sample placed on ice, and 1 ml of Feulgen reagent added at 5°C (section 2.2.5). The tubes were vortexed and then incubated for 2 h at room temperature after which the Feulgen reagent was removed and the stained tissue washed with 3 x 1 ml of SO_2-water for 3 x 15 min (section 2.2.6).

Slides were prepared and made permanent as follows: stained tissue was placed onto a slide in several drops of 25% acetic acid and firmly squashed beneath a silicon-coated, no. 2 cover slip (Chance Propper Ltd.), between filter paper. The slide was placed onto solid CO_2, to freeze, then the cover slip quickly prized off and the slide immersed in absolute alcohol, to dehydrate the tissue. After 5-10 min

the slide was removed and allowed to drain for 5-10 s. The tissue was preserved in 2-3 drops of 'Euparal' (G.B.I. Laboratories Ltd., BDH) under a no. 0 cover slip.

Preparation of standards

Diploid embryo and root tip tissues (Ellis et al., 1983) were processed alongside the endosperms to establish the relative fluorescence of 2C (G1) and 4C (G2) nuclei (Bennett, 1972; Bennett and Smith, 1976); using these values endosperm nuclei could be assigned to different ploidy classes (3C, 6C etc.). Embryos were collected from grains that had been fixed and dissected to yield endosperms. One cm root tips were obtained from 2 cm roots of wheat seedlings grown as described in section 2.2.1: they were stored in acetic acid : ethanol at 4°C. Standard tissues were rehydrated and slides prepared as described for endosperms.

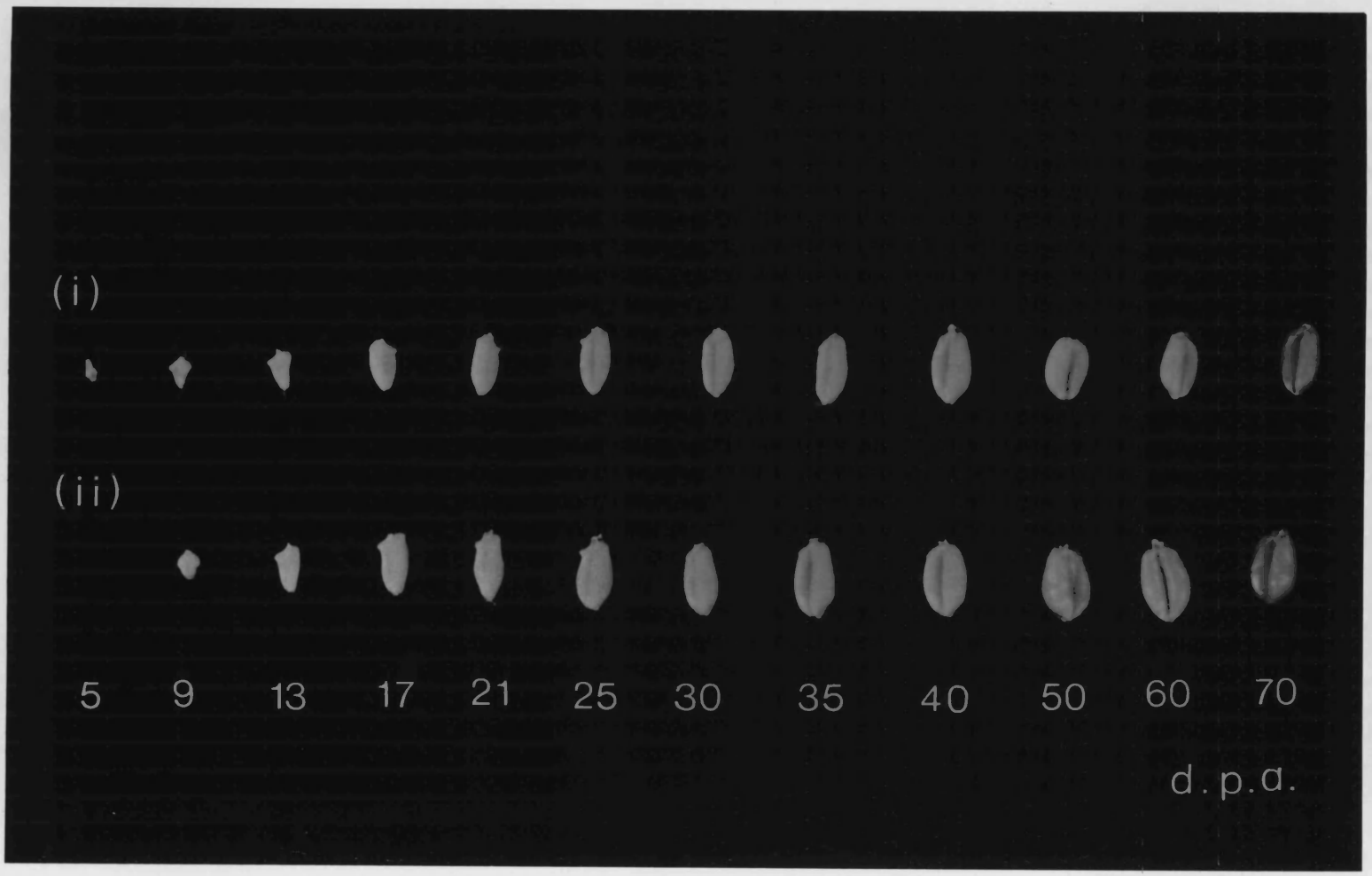
Fluorescence measurement and photography of stained endosperms

Nuclear fluorescence was measured as an estimate of DNA content (Böhm and Sprenger, 1968; Ruch and Rosselet, 1970). Fluorescence was measured using a Leitz MPV 3 microscope photometer using filter block N2 to provide excitation light at 530-560 nm. The instrument sensitivity was adjusted to ensure that large nuclei would not read off scale. Slides were viewed using tungsten light and fluorescence was measured for an integration time of 1 s. Single intact nuclei to be measured were magnified 500 times and located

within a 37 μm diameter measuring diaphragm. Nuclei were randomly selected by moving in a straight line across a slide. The clearest two slides for each sample were chosen and the fluorescence and backgrounds of 2 x 100 nuclei measured: background values were subtracted.

A Leitz Orthomat camera was attached to the microscope and photographs taken using Ilford XP1 film.

Figure 2.3 Wheat grains harvested at increasing days post anthesis and stored in acetic acid : ethanol
(i) C grains from intact spikes
(ii) C grains from degrained spikes


(i)
(ii)
5
9
13
17
21
25
30
35
40
50
60
70
d.p.a.

2.3 Results

Analysis of variance was employed to indicate whether means are significantly different. For data that were significantly different 95% confidence intervals for the difference between the means of two points of interest were calculated, using the student's *t* test, and where suitable, the pooled standard deviation.

Comparison of grains from mainstems and tillers

For grains from Batch 1 the data for fresh weight, dry weight, cell number and starch granule content (section 3.3) were recorded separately for mainstems and first tillers. However, it was consistently found that there were no significant differences between these two spikes at any of the developmental stages investigated here. For this reason the data were pooled and are presented as a whole throughout.

2.3.1 Grain fresh weight during development

Grains from floret positions A, B and C (from Batch 1 plants) showed a linear increase in fresh weight from 10 to 35 d.p.a., peaking at 50 to 60 d.p.a. (Fig. 2.3.1.1(i)). C grains initially developed about four days after the A and B grains, although their ensuing development was more rapid and at maturity the A, B and C grain fresh weights were not significantly different. D grains appeared to develop about five days after the C grains, attaining a maximum fresh

weight at 50 d.p.a. of 25.5±10% less than the C grain weight.

Degraining 'Timmo' wheat spikes resulted in very highly significant increases in fresh weight of the distal grains (Fig. 2.3): C grains from degrained spikes showing an increase over those from intact spikes of 40.0±10.4% at 50 d.p.a. and 45.8±15.7% at 60 d.p.a. (Fig. 2.3.1.1(ii)). D grains showed an increase of 61.0±13.8% at 50 d.p.a.: the D grains from degrained spikes grew slightly larger than the A, B and C grains from intact spikes, therefore these florets may possess the same potential at anthesis.

Batches 2 and 4, which were used for DNA extractions, were grown under similar growth cabinet conditions to Batch 1. Although Batch 2 C and D grains developed less rapidly, the significant differences between grains from intact and degrained spikes were comparable to those for Batch 1 grains: C grains from degrained spikes attained a peak weight at 60 d.p.a. that was 56.8±25.8% greater than that for intact spikes.

Batch 4 A grains developed slightly more rapidly than those from Batch 1 (Fig. 2.3.1.2). Grains from Batches 3.1 and 4 were harvested only over the linear phase of fresh weight increase: as expected, those that were greenhouse grown (Batch 3.1), with 3-37°C fluctuations developed earlier than those grown in a growth cabinet (Batch 4), with 10-15°C fluctuations (Wardlaw *et al.*, 1980).

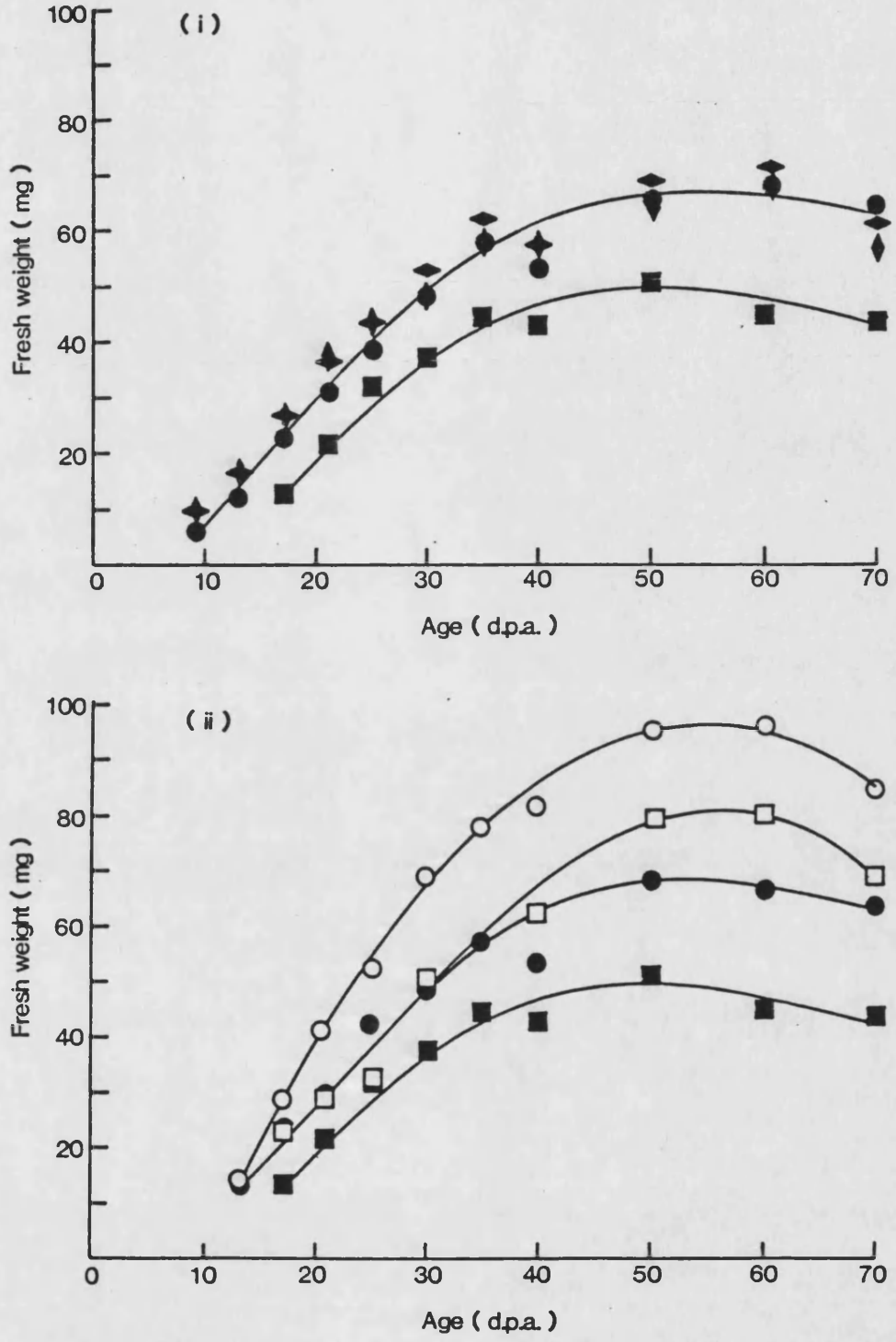


Figure 2.3.1.1 Fresh weights of grains (from Batch 1):
(i) A (◆), B (◆), C (●) and D (■) grains from intact spikes only
(ii) C and D grains from intact and degrained spikes: C intact (●); C degrained (○); D intact (■) and D degrained (□).
Means were calculated from 48 grains from two mainstems and two tillers.

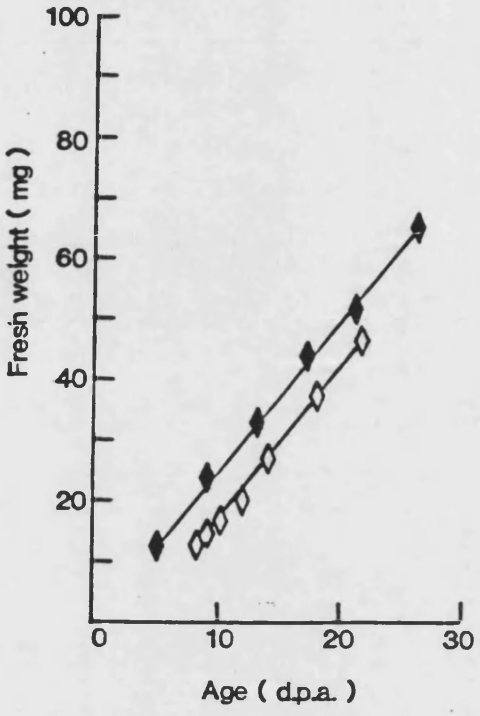


Figure 2.3.1.2 Fresh weight of A grains from Batches 3.1 (♦) and 4 (◊).
Means were calculated from between four and 40 grains.

2.3.2 Grain dry weight and water content during development

Curves of A, B, C and D grain dry weights (for Batch 1 plants) show similar relationships to those for their fresh weights, with the exception that dry weight reached a plateau in the mature grain whereas fresh weight fell as water was withdrawn (Fig. 2.3.2.1(i)). A, B and C grain dry weight peaks were attained by 60 to 70 d.p.a. At 60 d.p.a. the mean D grain dry weight was 37.6±12.7% less than the C grain weight.

C grains from degrained spikes showed increases in mean

dry weight above those from intact spikes of 47.5±10.5% at 50 d.p.a. and 32.8±12.7% at 60 d.p.a. The corresponding increases for D grains were 67.6±14.4 and 71.7±20.3%, respectively (Fig. 2.3.2.1(ii)). C and D grains from degrained spikes reached their maximum dry weight at only 50 d.p.a. indicating that they reached maturity earlier than those grains from intact spikes.

Total water content of C grains, from both intact and degrained spikes, reached a maximum at 30 d.p.a. and then fell from 50 to 70 d.p.a. to 55% and 65% of the maximum water contents, respectively (Fig. 2.3.2.2(i)). At 60 d.p.a. the water content of C grains from degrained spikes was 46.4±14.4% greater than for those from intact spikes. Percentage water content relative to grain dry weight fell from 370% at 5 d.p.a. to 45% at 70 d.p.a. (Fig. 2.3.2.2(ii)). Up to 40 d.p.a. the rate was virtually linear, after which the decline slowed down. Linear regression of the data for the two curves up to 40 d.p.a. showed that the y-intercepts, but not the slopes, were significantly different (y-intercept for grains from degrained spikes less that for those from intact spikes = 30.0±29.1%). It appears that the grains from degrained spikes have a slightly higher percentage water content: this may be because they develop sooner, although the difference is possibly maintained through to maturity.

Dry weight curves for A grains (from Batches 3.1, 3.2 and 4) are slightly sigmoidal, showing an increase in the rate of dry matter deposition with grain development (Figs. 2.3.2(i) and (ii)). The ages of A grains from Batch 3.2 were

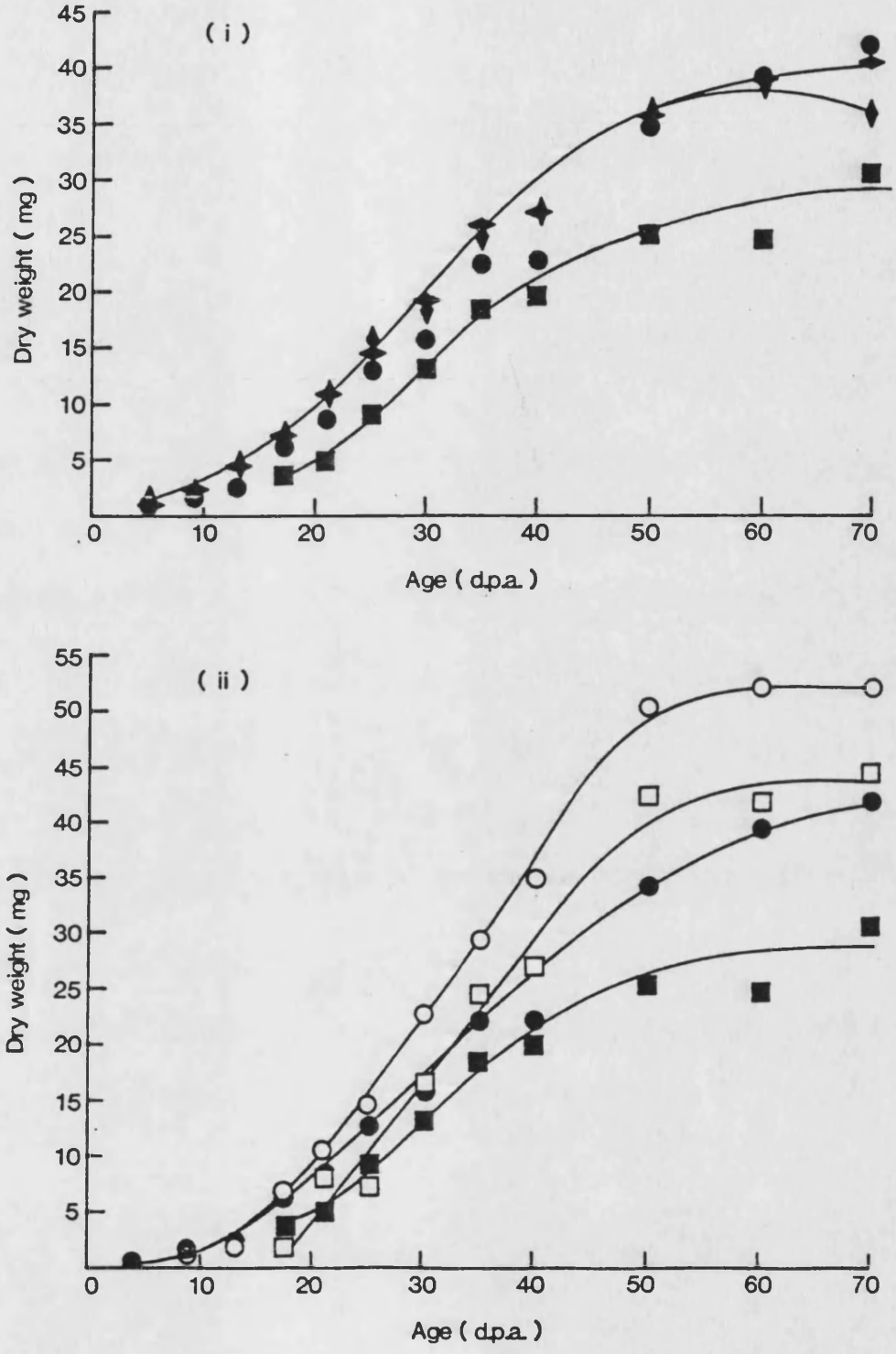


Figure 2.3.2.1 Dry weight of grains (from Batch 1):
(i) A (◆), B (◆), C (●) and D (■) grains from intact spikes only
(ii) C and D grains from intact and degrained spikes: C intact (●); C degrained (○); D intact (■) and degrained (□).
Means were calculated from 16 grains from two mainstems and two tillers.

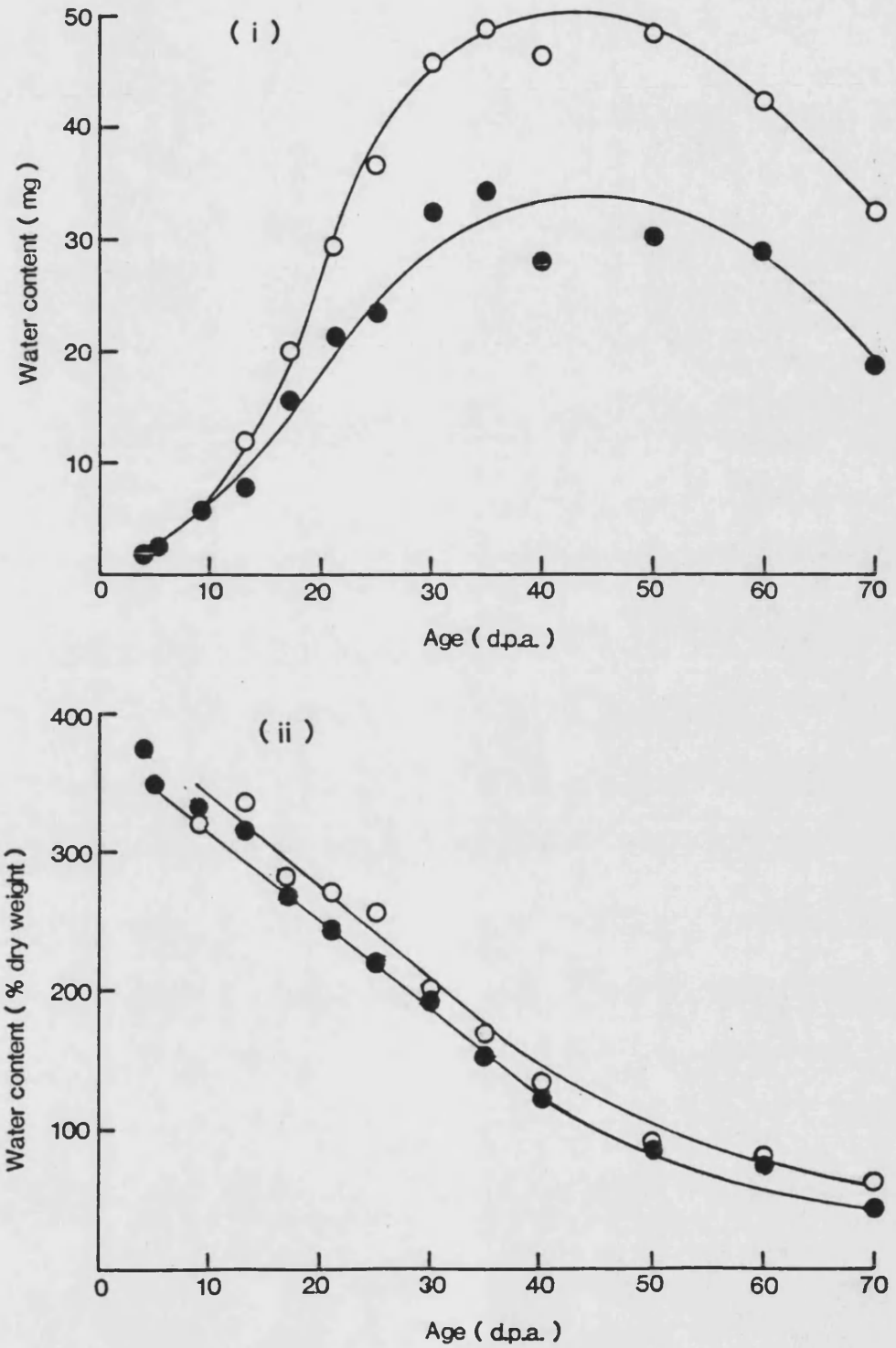


Figure 2.3.2.2 (i) Water content of C grains from intact (●) and degrained (○) spikes (from Batch 1)
(ii) Water content as a % of C grain dry weight: intact (■) and degrained (○) spikes.

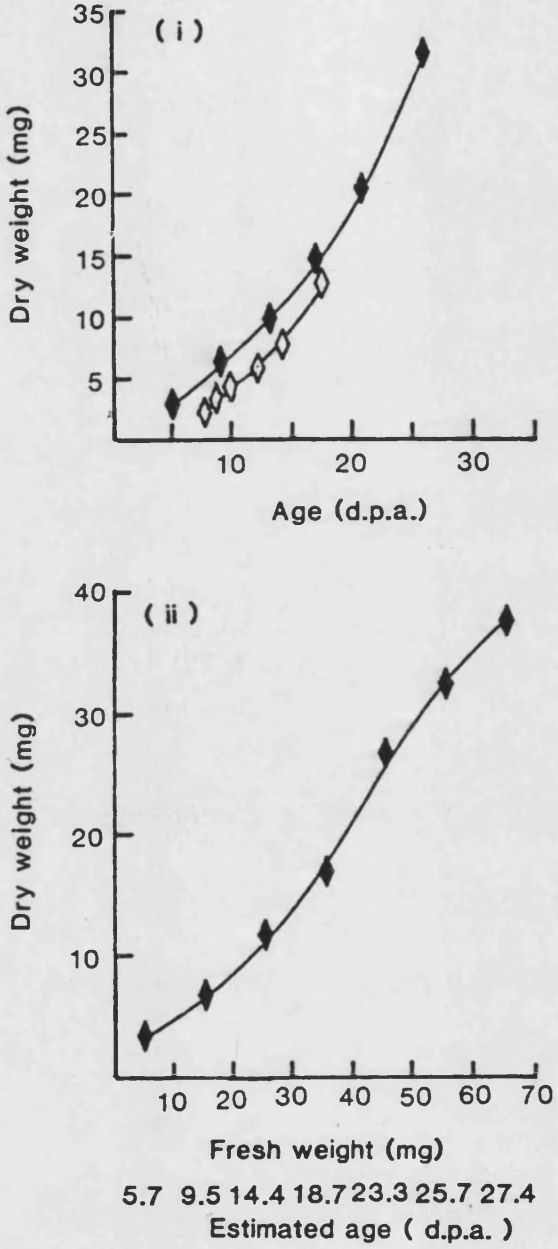


Figure 2.3.2.3 Dry weight of A grains from:
(i) Batches 3.1 (♦) and 4 (◊)
(ii) Batch 3.2 (♦)
Means were calculated from between two and eight grains.

estimated by comparing their dry weights with those for Batch 3.1. Total grain water content increased throughout the harvest schedule for all three batches of A grains. Percentage water content fell to 197% at 17 d.p.a. for Batch 4, 99% at 26 d.p.a. for Batch 3.1 and 100% at an estimated 27.5 d.p.a. (60-70 mg) for Batch 3.2.

2.3.3 Endosperm DNA content

DNA content of C grain endosperms (from Batch 1) increased linearly from 13 to 25 d.p.a., after which the increase slowed, reaching a maximum around 40 d.p.a. (Fig. 2.3.3.1). Degraining wheat spikes caused a mean increase in C grain endosperm DNA content of 53.4±16.8% for the period spanning 40 to 70 d.p.a. This is a notably greater figure than the 43.8% and 40.0% mean increase in fresh and dry weight, respectively, over the same period (Appendix V).

A grain endosperms (from Batch 3.2) showed a similar rapid increase in DNA content which was virtually linear up to 60-70 mg (27 d.p.a.)(Fig. 2.3.3.2).

2.3.4 Ploidy of endosperm nuclei

The percentage of endosperm nuclei in each of the ploidy classes, 3, 6, 12 and 24 C, was found to alter with grain age. For C grains (from Batch 1) the predominant ploidy from 9 to 13 d.p.a. was triploid, but by 21 d.p.a. the mean ploidy had increased and the majority of nuclei were hexaploid (Figs. 2.3.4.1(i) and (ii)). These trends are more clearly visible in the data for A grains (from Batch 3.2) where more

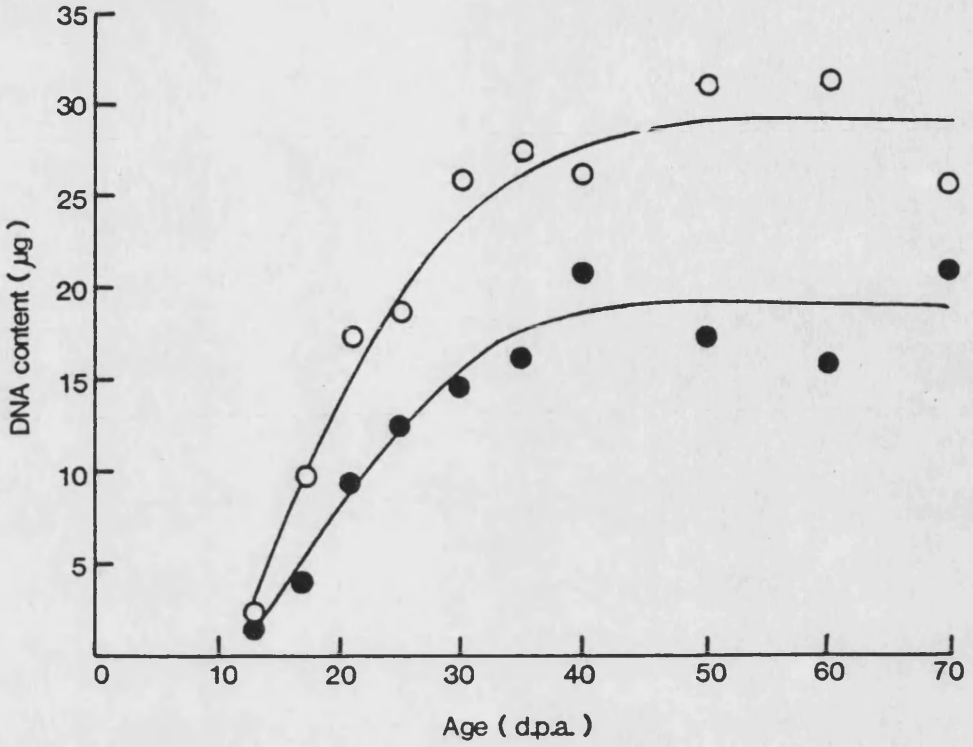


Figure 2.3.3.1 Total DNA content of C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1)
Means were calculated from between two and six replicate grains.

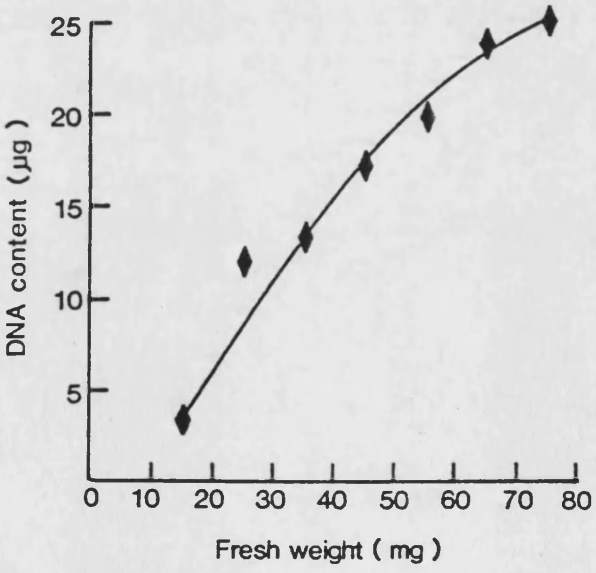


Figure 2.3.3.2 Total DNA content of A grain endosperms (from Batch 3.2).
Means were calculated from three replicate grains.

Number percentage in each ploidy
100
80
60
40
20
3 6 12 24
9
13
17
21
25
30
40
60
Age (d.p.a.)
(1)
Mean ploidy (C - value)
1
2
3
4
5
6

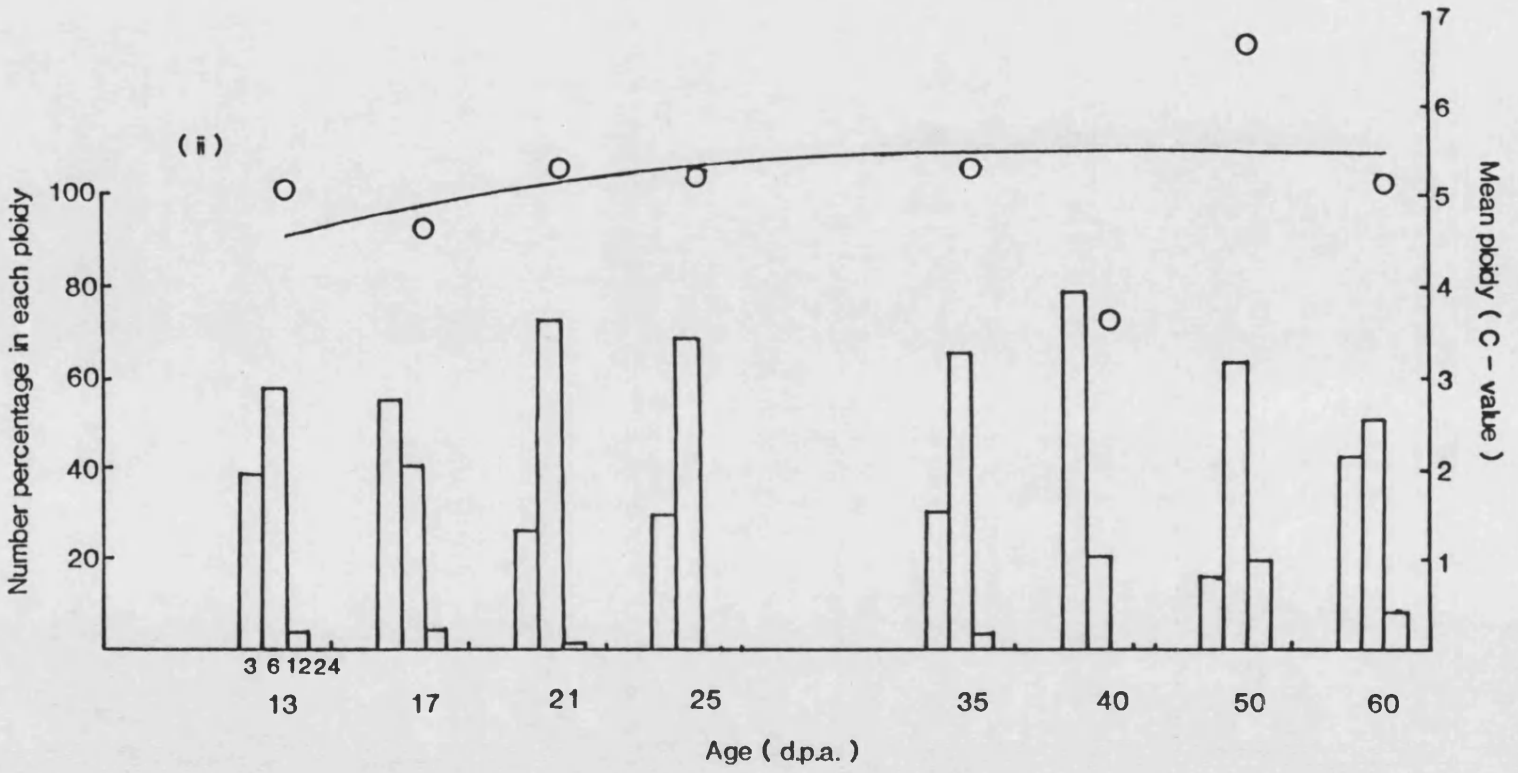


Figure 2.3.4.1 Microfluorimetry of Feulgen stained C grain endosperm nuclei from intact and degrained spikes (from Batch 1)

Histograms showing the percentage of nuclei estimated to be in the 3,6,12 and 24 C ploidy classes. Graphs showing mean ploidy were calculated from this data.

(i) C grains from intact spikes (●)

(ii) C grains from degrained spikes (following page) (○)

Only values up to 21 d.p.a. are means of two replicate grains.

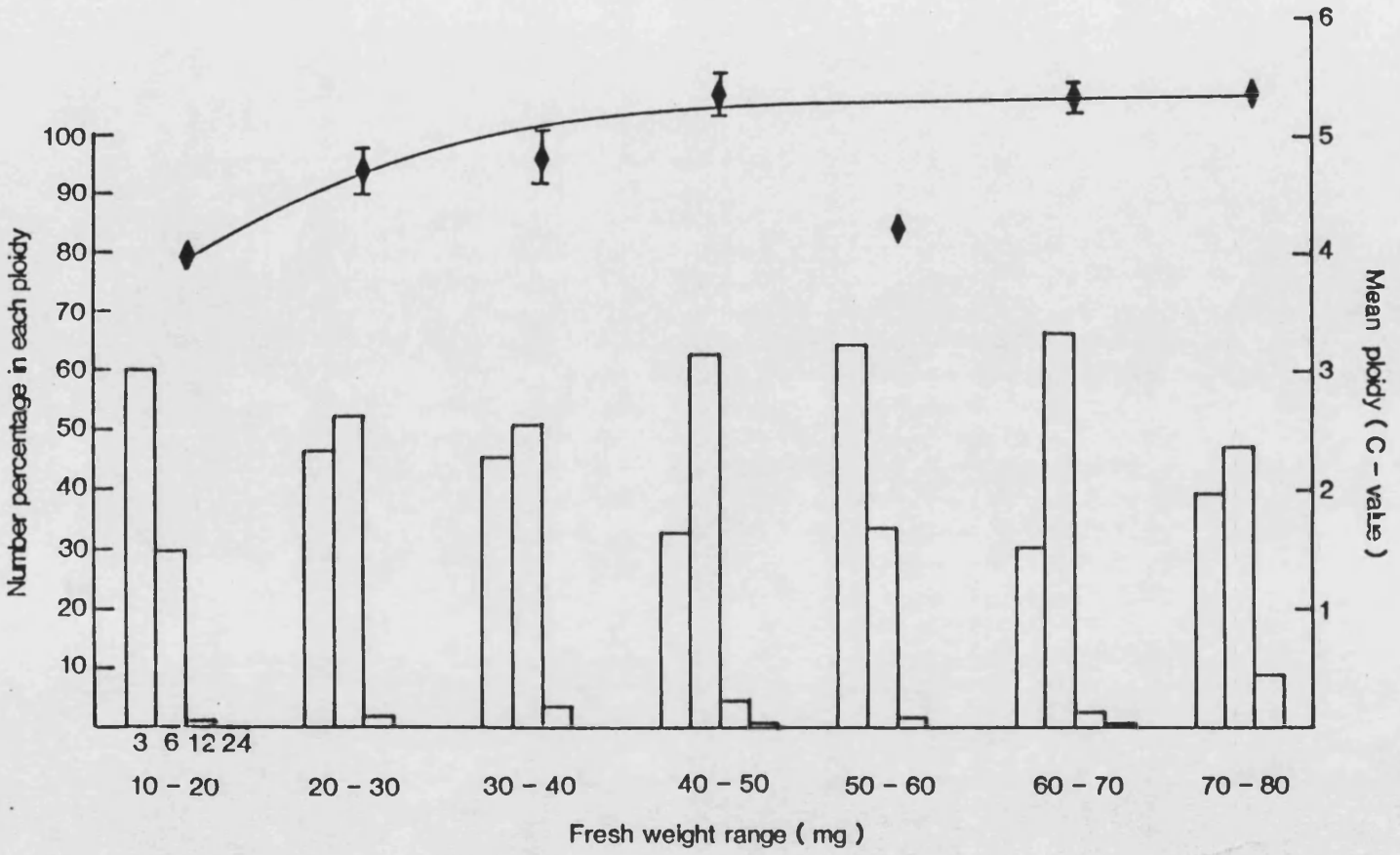


Figure 2.3.4.2 Microfluorimetry of Feulgen stained A grain endosperms (from Batch 3.2)
Histogram showing the percentage of nuclei estimated to be in the 3,6,12 and 24C ploidy classes.
Graph showing mean ploidy was calculated from this data.
Means were calculated from two replicate grains. Error bars show standard deviation.

replicates were assayed (Fig. 2.3.4.2). After 21 d.p.a. the number of 12 and 24 C nuclei detected appeared to increase only slightly in older grains.

Results proved to be quite variable, needing considerable replication for accuracy and, unfortunately, the technique is extremely time consuming. Erratic data points, especially from older grains, were ignored when drawing the curves for mean ploidy. For C grains from both intact and degrained spikes the mean ploidy increased from approximately 4.12 C, in the youngest endosperms, to a maximum of 5.65 C (Figs. 2.3.4.1(i) and (ii). For A grains a similar increase was seen: from 3.95 C at 9.4 d.p.a. (10-20 mg) to 5.35 C at 27.5 d.p.a. (60-70 mg). From these data it was not possible to detect any significant difference between the mean ploidies of C grain endosperms from intact and degrained spikes.

Comparison of the curves for endosperm mean ploidy and total DNA content, for both C and A grains, revealed that the average ploidy reached a constant value prior to the total DNA reaching a plateau. It would therefore appear that DNA synthesis continued for at least a few days without any change in ploidy being detected.

2.3.5 Photographs and analysis of Feulgen stained nuclei

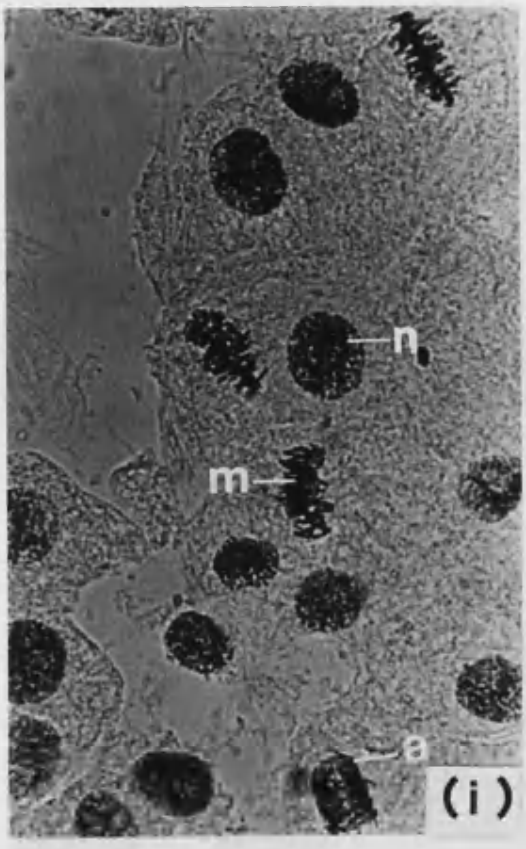
All photographs presented are of endosperm squashes prepared from Batch 1 C grains. Photographs of the youngest tissue, at 9 and 13 d.p.a., reveal large areas of nuclei in various stages of mitosis (Fig. 2.3.5.1(i)). These regions

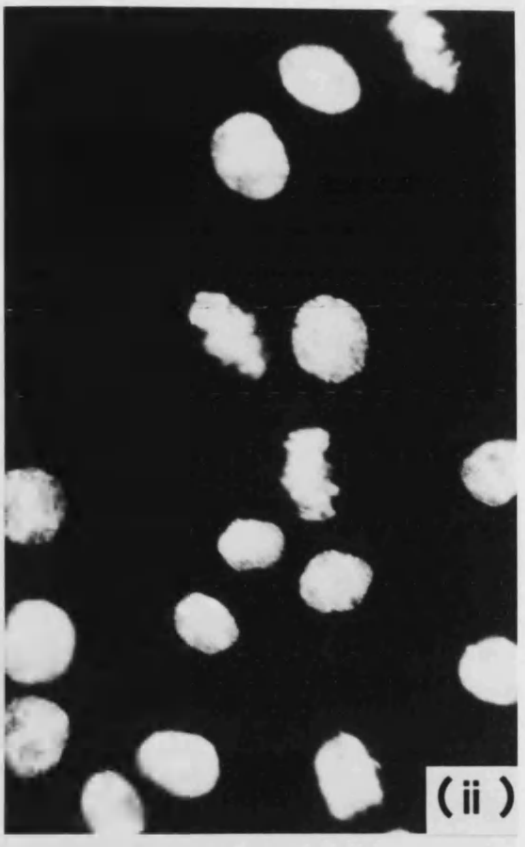
of nuclear and cell division are the primary reason for ploidy levels of more than 3 C in young endosperms. However, although prophase, metaphase, anaphase and telophase nuclei are all 6 C with respect to DNA content, fluorescence readings of less than the full 6 C complement were common (Fig. 2.3.5.1(ii)). It is possible that where the chromosomes are more compact, detection of DNA fluorescence by the microfluorimeter becomes less than linear.

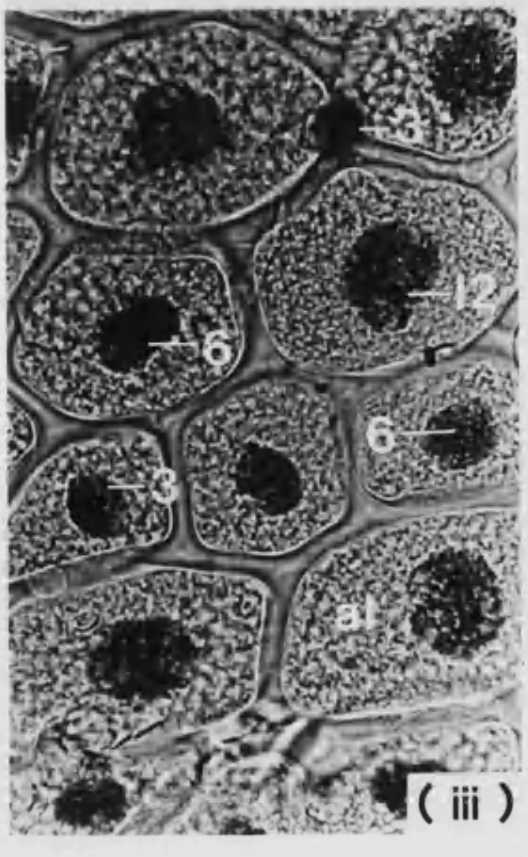
Aleurone cells were quite distinguishable in the more mature endosperms; with their thick cell walls and dense, granular cytoplasm, characteristically devoid of large starch granules (Sandstedt, 1946)(Figs. 2.3.5.1(iii)). Background cytoplasmic fluorescence was frequently greater in aleurone cells than in the larger, starch containing cells; although this was not evident in Fig. 2.3.5.1(iv). Like the starch containing endosperm, these cells show a range of nuclear ploidy, from 3 to 12 C, but 24 C nuclei were not observed.

As the endosperm develops and cell division slows, an increase in nuclear ploidy was not necessarily coupled to nuclear division, with the result that 6 C, 12 C and, very occasionally, 24 C nuclei were recorded (Figs. 2.3.5.1(iii), (iv), (v) and (vi)). Concurrently, starch granules were enlarging, occupying more of the cell and making it increasingly difficult to distinguish the purple-stained nuclei when using tungsten illumination (Fig. 2.3.5.4(v)). The nuclei were considerably more visible under fluorescent light (Fig. 2.3.5.4(vi), but despite this it is quite likely that starch granules reduced the levels of fluorescence

Figure 2.3.5.1 Feulgen stained endosperm nuclei
(i) Endosperm at 13 d.p.a. under tungsten light showing metaphase (m) and anaphase (a) nuclei (n)
(ii) The same view under ultra violet light
(iii) Aleurone cells (al) at 60 d.p.a. under tungsten light showing 3,6 and 12 C nuclei
(iv) The same view under ultra violet light
(magnification 40.2 μm)


n
m
a
(i)


(ii)


3
12
6
6
3
al
(iii)

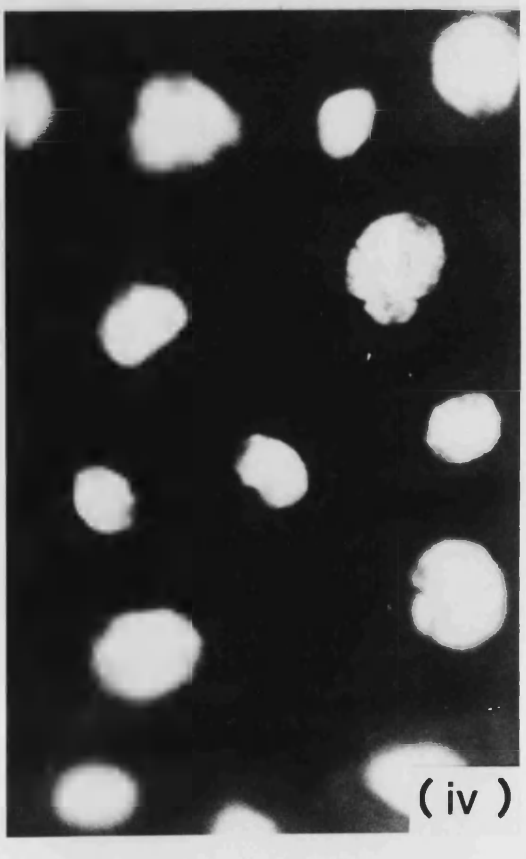
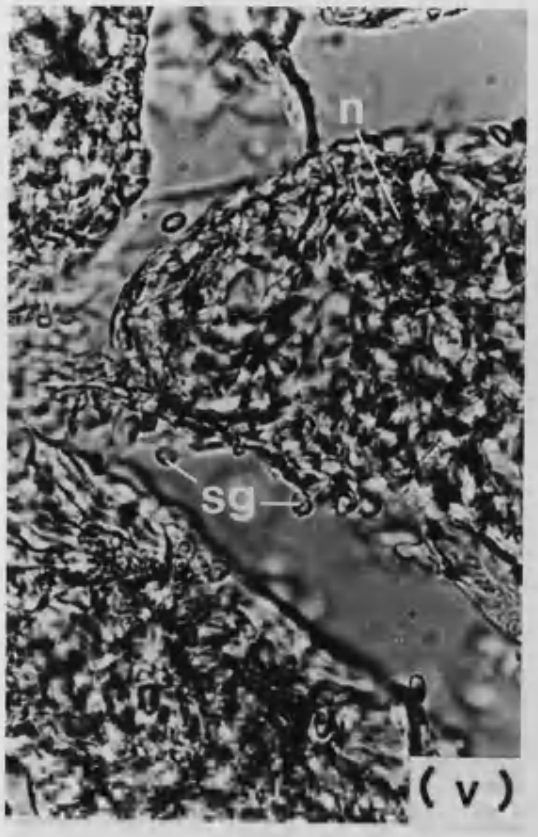
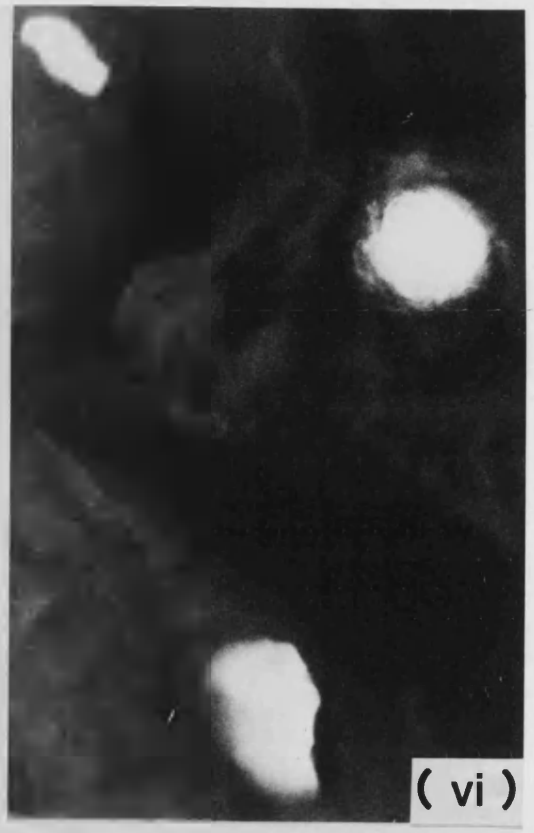

(iv)

Figure 2.3.5.1 Feulgen stained endosperm nuclei
(v) Endosperm at 40 d.p.a. under tungsten light showing starch granules (sg) obscuring nuclei (n)
(vi) The same view under ultra violet light, revealing nuclei much more clearly
(vii) Endosperm at 70 d.p.a. under tungsten light showing densely packed A (Ag) and B (sg) type starch granules
(viii) The same view under ultra violet light, showing a squashed nucleus (n) scarcely visible without fluorescence.
(magnification 40.3 μm)


n
sg
(v)


(vi)


Ag
sg
(vii)


n
(viii)

reaching the microfluorimeter. In endosperms of 60 to 70 d.p.a. the problem was compounded because the larger nuclei were often squashed into fragments, surrounded by starch granules, making them very difficult to measure (Figs. 2.3.5.1(vii), (viii)). This probably occurred during slide preparation.

2.3.6 Endosperm cell number

The curves for mean ploidy, in section 2.3.4, and for total DNA per endosperm, in section 2.3.3, were used to estimate mean cell number per endosperm. The 1 C DNA content was assumed to be 17.3 pg, as presented by Bennett and Smith (1976).

The results for C grains (from Batch 1) indicated that cell number increased rapidly from 13 to 25 d.p.a., after which the rate of cell division began to slow. From 40 to 70 d.p.a. the total endosperm cell number changed little, if at all (Fig. 2.3.6.1). Throughout grain development, C grain endosperms from degrained spikes contained a markedly greater cell number, compared with those from intact spikes: from 40 to 70 d.p.a. the mean increase was 53.4%. This large difference was entirely due to the different DNA contents of the two grain types because the mean ploidy values were taken from identical curves. Comparison of endosperm DNA content (Fig. 2.3.3.1), with endosperm cell number, for both degrained and intact spikes, reveals that the two curves could virtually be superimposed: both reach a plateau by 40 d.p.a., after an almost linear increase from 13 to 25 d.p.a.

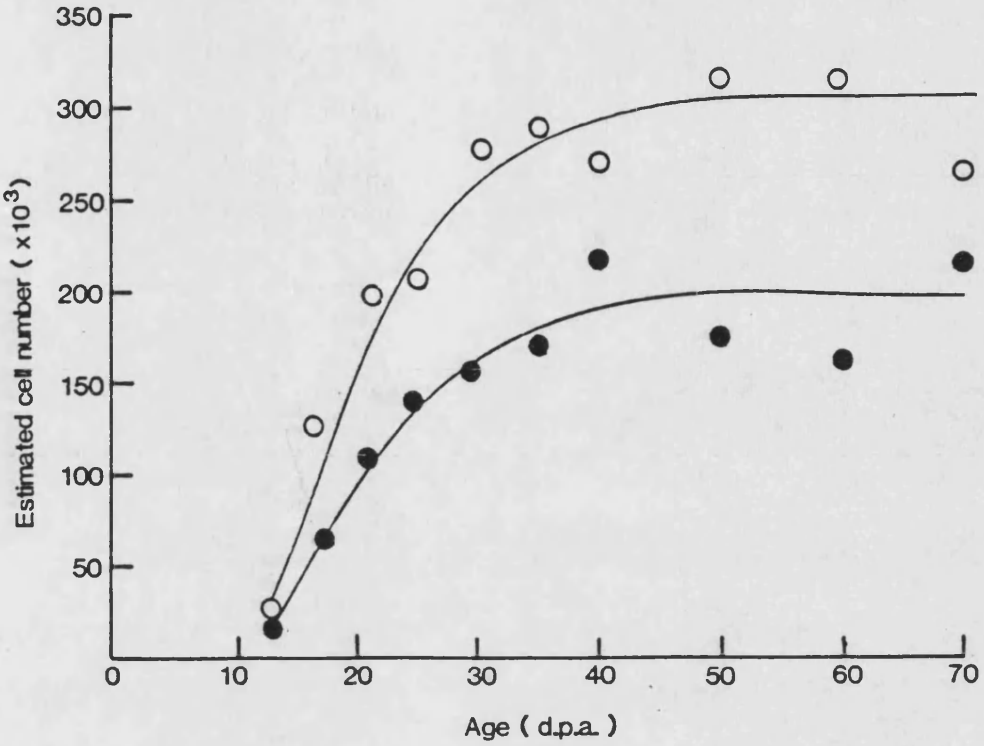


Figure 2.3.6.1 Cell number of C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1), as estimated from grain DNA content, mean nuclear ploidy and a 1 C value = 17.3 pg (Bennett and Smith, 1976)

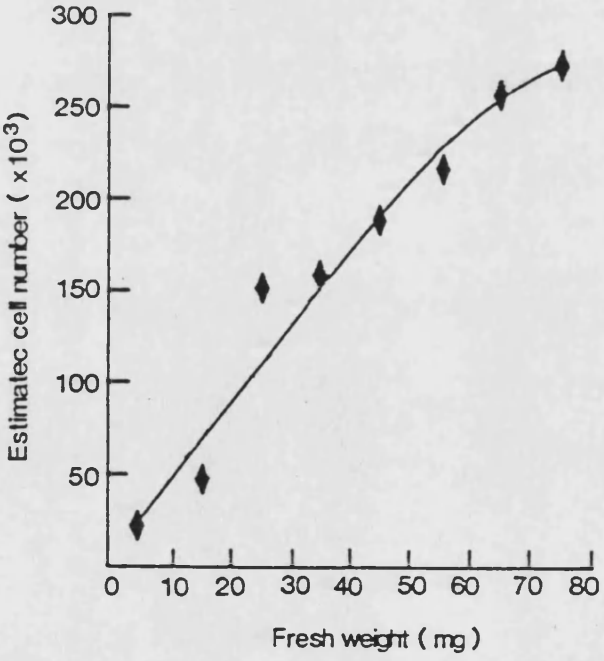


Figure 2.3.6.2 Cell number of A grain endosperms (from Batch 3.2) estimated as for Fig. 2.3.6.1.

A grain endosperms (from Batch 3.2) showed a linear increase in estimated cell number from 5.7 to 25.7 d.p.a. (0-10 to 50-60 mg), after which the increase began to slow (Fig. 2.3.6.2). The curve for cell number parallels the increase in A grain endosperm DNA content; both continuing to increase long after mean cell ploidy had reached a plateau (Fig. 2.3.4.2)

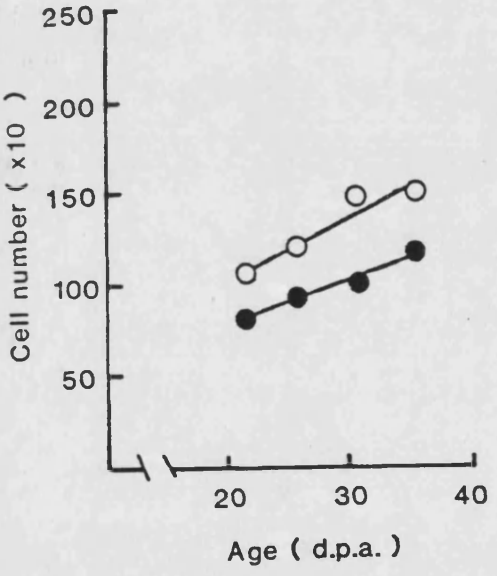


Figure 2.3.6.3 Cell number of C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1), as determined by counting Feulgen stained nuclei.

Cell number was also determined by counting Feulgen stained nuclei on Millipore filters. The results (for C grain endosperms) presented in Fig. 2.3.6.3, cover the period from 21 to 35 d.p.a. and confirm that there is a significant increase in cell number of endosperms from degrained spikes, relative to those from intact spikes, of 34.1±15.4%. However, these cell number figures are considerably lower than the equivalent values estimated from the mean ploidy and endosperm DNA content data. When determined by direct counting, mean cell numbers for C grain endosperms from intact and degrained spikes were only 76.3% and 53.9%, respectively, at 21 d.p.a. and 68.7% and 51.5%, respectively, at 35 d.p.a., in comparison with the previous, estimated values. The discrepancy between the two data sets increases with grain age.

2.3.7 Estimated mean cell dry weight

Endosperm cell number, estimated from mean ploidy and total DNA content (Figs. 2.3.6.1 and 2.3.6.2), and grain dry weight data were used to give a rough estimate of mean cell dry weight. The results can only serve as a crude comparison since endosperm dry weight, as opposed to grain dry weight ~~data~~, were not available: for this reason, figures were only determined for grains of more than 15 mg dry weight, since for smaller grains the pericarp tissue constituted a more considerable proportion of the total grain (Evers, 1970,1974). Inevitably all results are slightly overestimated, and more so for the younger grains; however,

it was considered that since starch comprises 70-80% of the dry weight of the mature grain (Jenner, 1982a) and as most of this starch is located in the endosperm (Simmonds and O'Brien, 1981), the results should give a good indication of relative cell dry weights in maturing endosperms.

Comparison of curves for C grains from intact and degrained spikes (from Batch 1) from 30 to 70 d.p.a. indicated that mean cell dry weight for endosperms from degrained spikes was an average 19.6±10.8% less throughout this period (Fig. 2.3.7.1). In fact, if data for the period spanning from 13 to 70 d.p.a. is considered a difference of 19.6±13.7% was maintained. To what extent this was effected by endosperm starch content is examined in Chapter 3.

A grain endosperms showed a similar increase in mean cell dry weight although this had not reached a plateau by the final sampling date (Fig. 2.3.7.2).

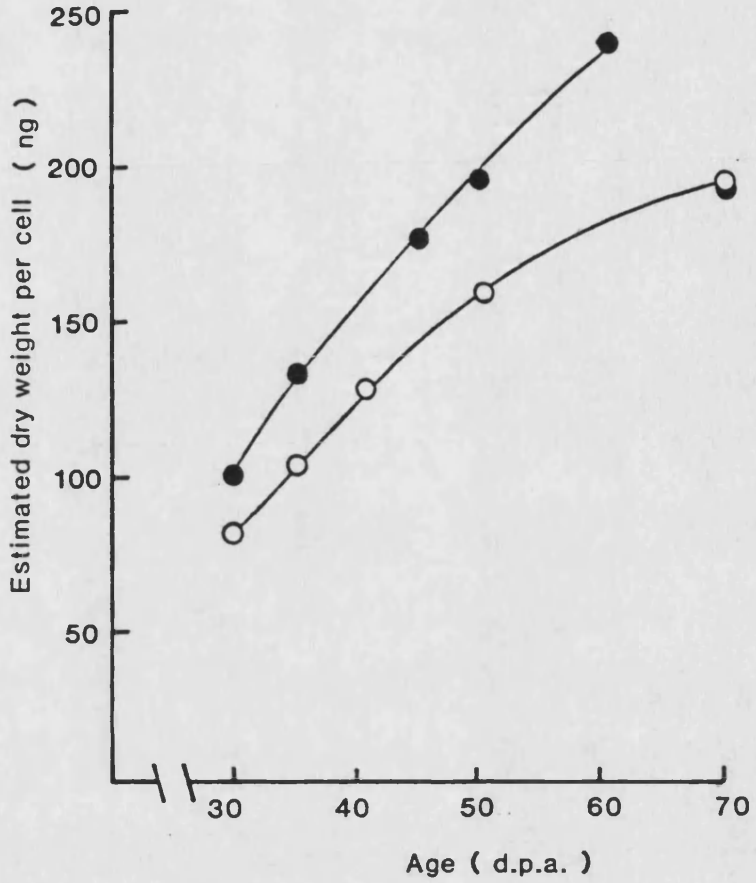


Figure 2.3.7.1 Estimated dry weight per cell for C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1).

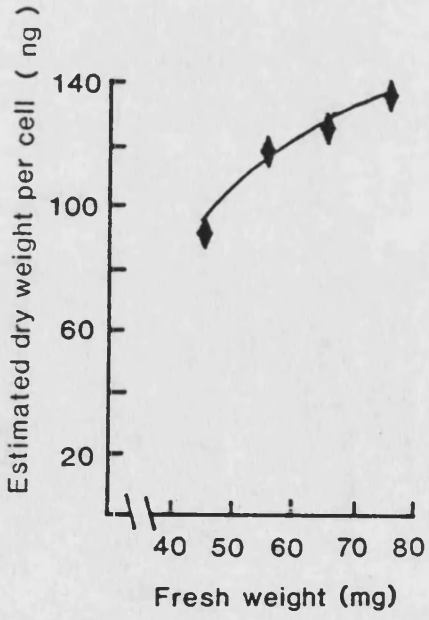


Figure 2.3.7.2 Estimated dry weight per cell for A grain endosperms (from Batch 3.2).

2.4 Discussion

2.4.1 Grain growth potential

The order of grain development in intact spikes was sequential from A through to D (A $\geq$ B > C > D), but final grain weights revealed the more rapid growth rate of C grains since, at maturity, A, B and C grain fresh and dry weights were not significantly different (A $\simeq$ B $\simeq$ C >> D)(Figs. 2.3.1.1 and 2.3.2.1). This is in accordance with other published data (Rawson and Evans, 1970; Bremner, 1972), although different orders of grain size have been reported (B > C > A > D and A $\simeq$ B > C > D) which have been attributed to more optimal growth conditions rather than genotypic differences (Bremner and Rawson, 1978). Mature grain weight was determined by the duration as well as the rate of growth (Herzog and Stamp, 1983; Gleadow *et al.*, 1982), since more distal grains reached anthesis later (Evans *et al.*, 1972).

Sterilisation of the basal florets revealed that the distal C grains, of Timmo wheat, have the capacity to accumulate up to 40.0% more dry matter, the mean increase from 40 to 70 d.p.a. (Fig. 2.3.2.2), although the difference at maturity was less at 24.4%. In comparable degraining experiments using the cultivars Hobbit (Radley and Thorne, 1981) and Kaiser (Radley, 1978) similar increases in C grain dry weight were detected: of 40% and 25-37%, respectively. In contrast, Bremner and Rawson (1978) found that C grain size in central spikelets of a Mexican semidwarf variety actually decreased after degraining and a smaller dry weight increase of only 11% was found for Maris Huntsman (Radley and

Thorne, 1981). Although these differences may reflect varietal constraints (Radley and Thorne, 1981) they may in other instances be ascribed to different growth conditions: where these are optimal the control C grains from intact plants may attain more of their yield potential thus reducing the unexploited capacity (Radley, 1978; Bremner and Rawson, 1978).

The increased fresh and dry weight of the C and D grains, after degraining, was a result of more rapid rather than a longer duration of growth; in fact the latter may be curtailed sooner in the larger grains. That increases in rate, as opposed to duration, primarily contributes to greater grain size, within a variety, is in accordance with previously published work (Herzog and Stamp, 1983).

The distal D grains showed a greater dry weight improvement than C grains after degraining: a mean increase of 57.3% being attained from 40 to 70 d.p.a. The degraining experiments of Bremner and Rawson (1978) also showed considerably greater increases in D grain fresh weights compared with the changes in C grain weight. In the experiments presented here the D grains did not attain the same size as the C grains from the same degrained spikes. It may be that the C grains limited their development, assumably via the same process as that by which the C and D grains are limited normally by the A and B grains. Alternatively it may be that although their unexploited capacity was greater than that of the C grains, their overall yield potential may be less. If the latter is true this may be a result of their

later initiation (Figs. 2.3.1.1 and 2.3.2.1) or their distance from the rachis (Hanif and Langer, 1972): however, A grains which are most favourably situated, are often no larger or even smaller than the more distal B and C florets (Bremner and Rawson, 1978). A restricted yield potential of the A grains would appear to be consistent with evolutionary advantage (section 1.3) since the limited potential and proximity to the rachis ensures A grain growth when assimilate is scarce, yet limits its interference of more distal grains when assimilate is in better supply.

It appears that the yield potential of C grains, at least for certain wheat varieties (cf. Bremner and Rawson, 1978), may be greater than that of A grains, as evidenced by the greater ability of the C grains to compete for assimilate (Cook and Evans, 1983). However, the degree to which the C grain potential is fulfilled may be less, thus the unfulfilled capacity of these grains is great and likely to increase by a greater proportion than for A and B grains as the growth conditions become less optimal (Bremner and Rawson, 1978).

2.4.2 Effect of grain removal on grain water content

The apparent increase in the percentage water content of C grains from degrained spikes is in accordance with previous data: Radley (1978) found that total grain water content increased more than did the total grain dry weight. She describes that this was due to an enlarged endosperm cavity in the larger grains which contained more than a five fold

greater fluid volume; the sucrose concentration of which was not significantly different ($P \leq 0.01$). From this data it is not possible to tell whether the cell percentage water content is altered by grain removal.

It appears that dry matter deposition is not necessarily prevented by the percentage grain water content falling to a particular level since in C and D grains from degrained spikes grain weight increase ceased as early as 50 d.p.a. when the water content, as a percentage of grain dry weight, was at least 90%; whereas in intact spikes dry matter deposition continued until 70 d.p.a. when water content was approximately 45% relative to the grain dry weight. The process causing grain filling to terminate is not known but it would appear that the deposition of lipids in the chalazal region, which has been suggested to seal off the pathway of water and nutrient flow (Zee and O'Brien, 1971; Sofield *et al.*, 1977b) is unlikely to be the primary cause (Nicolas *et al.*, 1984).

2.4.3 Effect of grain removal on endosperm DNA content

The data for endosperm DNA content at maturity was compared with published data for other wheat varieties (Fig. 2.4.3). This revealed that mature C grains from intact and degrained spikes appeared to fall within the scatter of ratios of grain DNA content to endosperm or grain dry weight ratios recorded previously, for grains from unspecified floret positions (Donovan, 1979, 1983; Chojecki *et al.*, 1986a). Linear regression of these data reveals that for

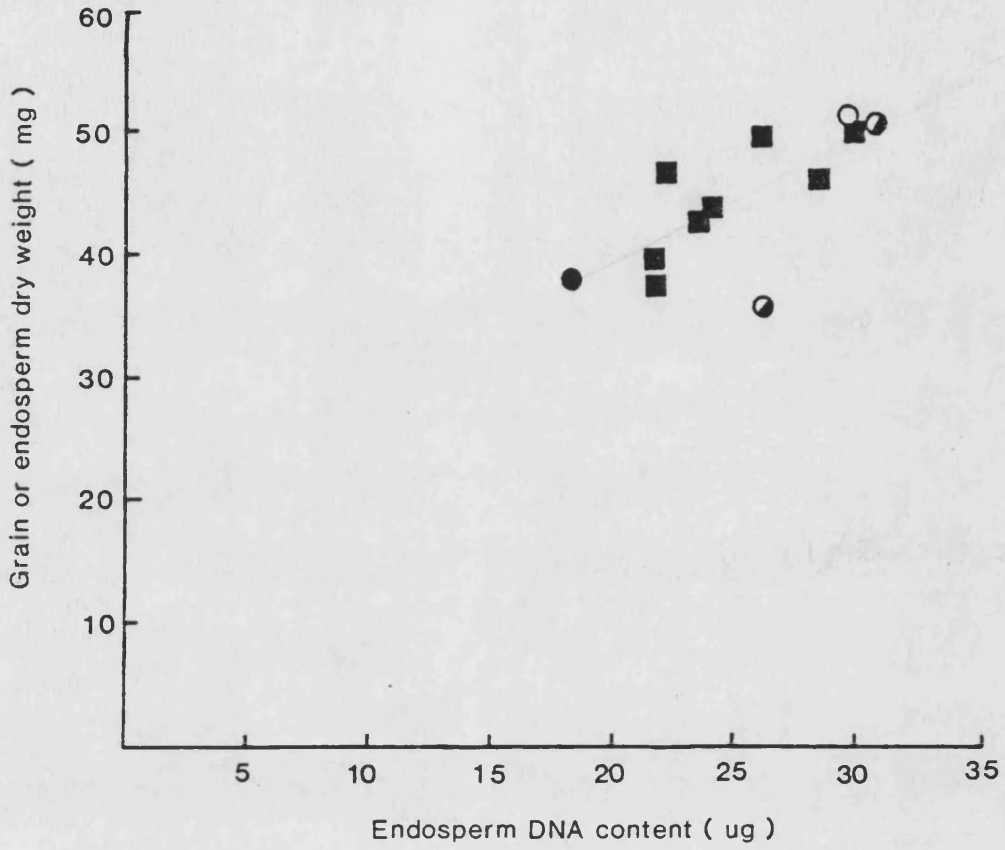


Figure 2.4.3 Grain or endosperm DNA content versus grain dry weight at maturity. Figures were calculated from mean DNA content and dry weights for grains where dry weight had reached a plateau: data from Chojecki et al. (1986a) and Chojecki (1985) (◒), data from Donovan (1979) (■) and results for C grains from intact (●) and degrained (○) spikes (from 50 to 70 d.p.a., this thesis).
(◒,○,● were calculated from data for endosperm DNA content and ■ from data for grain DNA content).

mature grains the ratio does not appear to be 1:1, but nearer 1:0.75, with a two fold greater final DNA content being associated with a 1.5 fold greater mature grain dry weight. The implications of the 1:0.75 ratio are that larger grains either comprise cells containing less dry matter or of a higher cell ploidy or a little of both. Although each of the points on the graph represent different wheat cultivars, with the exception of those from this thesis, further data would help to determine how accurate and universal this trend is and whether the same pattern is found for grains of a particular cultivar grown under different environmental conditions.

A comparison of the fresh and dry weight and endosperm DNA content curves for C grains from intact and degrained spikes indicates that the relatively lower increase in grain weight compared with grain DNA content is not only seen at maturity but also throughout the development of these grains. Comparison of the mean percentage increases for endosperms from degrained spikes compared to intact spikes, from 9 to 70 d.p.a., revealed that the relatively greater increase in DNA content was significant; the percentage increases being 32.6 ± 9.3 and 27.1 ± 13.6 for grain fresh and dry weights, respectively and 67.0 ± 18.7 for endosperm DNA content.

2.4.4 Feulgen staining procedure and ploidy of endosperm nuclei

For many plant species cell differentiation is regularly accompanied by nuclear endoreduplication cycles (Barlow,

1985; Nagl, 1978) where DNA is reproduced but there is no nuclear or cell division. As a consequence the nuclei become endopolyploid or polytene, depending on whether or not the replicated chromatids separate (Nagl et al., 1985), and the cells usually become greatly enlarged (Cavalier-Smith, 1978). The existence of endopolyploid nuclei in wheat endosperm (Chojecki et al., 1986a) has been previously reported and similar nuclei have been found in the endosperms of other cereals (Lin, 1977; Cosgrove Keown et al., 1977; Maherchandani and Naylor, 1971). The functional signficance of endocycles has been investigated (Nagl et al., 1985) and it is considered that these may enable the cell to undergo more rapid transcription, which is of signficance for highly specialised, protein and starch storage cells such as the endosperm (Müntz, 1978); cell size may increase and the relationship between genes and non-coding DNA may be altered, with a possible effect on gene regulation.

The Feulgen fluorescence measurements of nuclei, especially from grains older than 13 d.p.a., confirmed the presence of nuclei of a wide range of sizes and levels of fluorescence in endosperm cells. These nuclei did not fall within narrow fluorescence channels indicative of the 3, 6, 12 and 24 C ploidy classes but covered a broad distribution spanning from less than 3C to as high as 24C, as indicated by root tip standard 2 and 4C nuclei. Cosgrove Keown et al. (1977) obtained absorbance measurements of Hordeum vulgare aleurone nuclei which similarly spanned ploidy classes of less than 3C up to around 12C. They were totally unable to

locate 3, 6 and 12 C peaks and the major peaks obtained appeared to shift during development (see also Galli et al., 1986). They discuss that this might be due to the amplification of specific cistrons (Heidecker and Messing, 1986), as had been suggested previously by Maherchandani and Naylor (1971), or the presence of megachromosomes (Bennett and Smith, 1976), or the under-replication of certain sequences (Nagl et al., 1985), as suggested for Pisum sativum polyploid cells (van Oostveldt and van Parijs, 1976). However, Millerd and Whitfeld (1973) claim that endoreduplication involves replication of the whole genome in Vicia faba cotyledons and analogous results were presented for pea cotyledons (Müntz, 1978). Furthermore, Bennett and Smith (1976) argue that despite these sources of variation the cell DNA content tends to remain quite constant (±3-5%). Lin (1977) found maize endosperm cells with a 9C complement of chromosomes which he attributed to fusion of 3C and 6C nuclei after irregular cell plate formation at the coenocytic stage of development.

Similar difficulties were encountered with the fluorescence measurements obtained in this project with respect to assigning nuclei to distinct 3, 6, 12 and 24C categories. It is therefore suggested that the histograms presenting DNA ploidy (Figs. 2.3.4.1-2.3.4.2) should be considered as indicating overall trends rather than absolute proportions of nuclei in each ploidy class. Clearly the measured nuclear DNA content will vary not only as a result of endoreduplication, but also depending on the proportion of

nuclei at different stages of the cell cycle (McLeish and Sunderland, 1961) since endosperm cell division is not synchronous (Bennett et al., 1975) and DNA replication is not instantaneous (Yeoman, 1981). In addition, it is claimed that nuclear DNA does not always stain evenly: highly polymerised or amplified DNA may stain less and extremely dense DNA and heterochromatin may result in underestimates (Ruch and Rosselet, 1970; Verma and Rees, 1974; Bennett and Smith, 1976). Particularly dense nuclei have been observed in the dividing cells of young endosperm tissue (this thesis and Bennett et al., 1973): similarly small, probably mitotic, nuclei were observed in young maize endosperms (Lin, 1977).

Problems were also encountered due to the large numbers of starch granules which either obscured or squashed the nuclei of more mature grains (Chojecki, 1985); furthermore the nuclei of mature endoperms have been found to be lobed (Briarty et al., 1979). It is probable that fluorescence, as opposed to spectrophotometric absorbance measurements, of the obscured nuclei should detect more of the Feulgen stain (Bennett and Smith, 1976). Although no comparison was made here of the advantages of fluorescence over spectrophotometric measurements of Feulgen stained nuclei, it has been suggested that the former should be more sensitive (Böhm and Sprenger, 1968; Yeoman, 1981).

Other reports have revealed problems caused by background fluorescence of the cytoplasm (McLeish and Sunderland, 1961) which has been particularly severe when aleurone tissue has been examined, despite aldehyde blocking

(Cosgrove Keown et al., 1977). For the most part, background fluorescence of endosperm cells was low when the hydrolysis and staining procedure described in section 2.2 was followed; although both aleurone and embryo tissues retained stain in some preparations, probably where hydrolysis was not sufficiently severe to penetrate to cytoplasmic RNA (Bennett and Smith, 1976). Clearly the resistance of various plant tissues to hydrolysis varies both within and between species making absolute measurements prone to error; but it is critical that a standardised hydrolysis treatment should be adopted if the Feulgen stain is to be quantitative (McLeish and Sunderland, 1961; Bennett and Smith, 1976). It may be that the tissues could be made more standard prior to staining, by cellulase, amylase (Singh and Jenner, 1982; Gleadow et al., 1982) and RNase (Carlberg et al., 1984) treatments, provided neither nuclear integrity nor stain complexing was adversely affected, although this would preclude the determination of nuclei number per cell (see Ellis et al., 1983).

Despite possible limitations of the Feulgen fluorescence and spectrophotometric absorbance techniques with respect to assigning individual nuclei to particular ploidy categories, a comparison of changes in mean tissue ploidy is likely to be reasonably accurate, particularly for younger endosperm tissues. Throughout endosperm development this mean was considerably greater than the 3C content of the first triple fusion nucleus. Initial increases were probably largely due to the mitotic S phase of DNA replication (Lin, 1977;

Yeoman, 1981) and further increases in mean ploidy, in this work up to between 5.2C to 5.6C, for all three Timmo grain types examined (Figs. 2.3.4.1-2.3.4.2), have been attributed to endoreduplication (Chojecki et al., 1986a; Lin, 1977; Cosgrove Keown et al., 1977). There was no significant difference between the data for C grains from intact and degrained spikes, although the ploidy of the former may have been slightly greater. In the cultivars Chinese Spring and Spica these mean figures were nearer 6.8C and 6.2C, respectively (Chojecki et al., 1986a), largely due to a slightly greater increase in the percentage of 24C nuclei.

Increases of the same or a slightly greater magnitude have also been recorded for barley endosperm (Cosgrove Keown et al., 1977) and other plant storage tissues, including Solanum tuberosum tuber (Jacobsen et al., 1983) and bean and pea cotyledons (Millerd and Whitfeld, 1973; Davies, 1976). Although little has been done to determine the spatial and temporal position of these nuclei in storage tissues, including the endosperm, there has been a comparable study on maize root xylem tissue (Barlow, 1985). Barlow determined that each endoreduplication phase (endo-S) was separated by a non-DNA synthetic phase (endo-G) and these occurred in zones at fixed distances from the meristem. Together, data presented by Barlow and others (Bennett et al., 1973, 1975; Yeoman, 1981) indicates that increased ploidy, within a species, does not necessarily increase the endo-S phase relative to diploid cells, although the endo-G phase can vary in duration.

2.4.5 Endosperm cell number and the effect of grain removal

Determination of endosperm cell number from the mean cell ploidy and total DNA content data gave maxima of approximately 180 and 300 x 10^3 for C grains from intact and degrained spikes, respectively (Fig. 2.3.6.2). Chojecki et al. (1986a), using the same technique, obtained maxima of 240 and 280 x 10^3 cells per endosperm for the cultivars Chinese Spring and Spica, respectively. These figures exceed earlier estimates determined by direct counting of Feulgen stained nuclei (Rijven and Wardlaw, 1965): Wardlaw (1970) counted 70 x 10^3 and Brocklehurst (1977) between 122-145 x 10^3. However it was found in this work (Fig. 2.3.6.3) and also by others (Chojecki et al., 1986a; Singh and Jenner, 1982a; Gleadow et al., 1982) that the direct counting procedure is limited by the increasing numbers of starch granules which obscure the nuclei of endosperms of increasing age. Singh and Jenner (1982a) therefore attempted to increase the counting efficiency by enzymatic digestion of the endosperm starch, using α-amylase, and the resultant improvement in the number of nuclei observed gave maximum counts of 165 x 10^3 (Singh and Jenner, 1982a,b) and of up to 200 x 10^3 (Singh and Jenner, 1984), for grains from detached spikes provided with higher concentrations of glutamine and sucrose or high irradiance. In comparison, Gleadow et al. (1982), using a similar protocol, obtained estimates of only 70-105 x 10^3 cells per endosperm in grains from basal florets of six cultivars (see also Nicolas et al., 1984, 1985). It was also found that, despite starch digestion, cell number appeared to

fall by up to one third (Singh and Jenner, 1982a) or more (Gleadow et al., 1982) as the grains matured. In contrast, endosperm cell number estimates from the ploidy and DNA content data showed only a slight decrease in cell number at maturity (Chojecki et al., 1986a), or none at all (this thesis).

However, this procedure may, like direct counting, be prone to error: either an overestimated DNA content or an underestimated mean ploidy would result in an erroneously high estimate. Since starch granules did pose a problem with the Feulgen fluorescence technique (section 2.4.4), it would appear that any inaccuracy may lie here, although no significant decline in ploidy was detected in more mature endosperms.

Estimates of cell number from stereological analysis of endosperm tissue also gave values lower than those calculated by this method: 92.4 and 152.8 x 10^3 for C grains from intact and degrained spikes, respectively (Radley, 1978) and 110 x 10^3 for unspecified grains (Briarty et al., 1979). It may therefore be that the true values fall somewhere between those given by the different data available. However, differences between reports with respect to variety (Gleadow et al., 1982), growth conditions (Singh and Jenner, 1982b, 1984) and grain position within the spikelet or spike may account for much of the variation in estimated cell number.

Final differences in cell number estimates within an experiment were the result of differences in both the rate and duration of cell division, which appeared to be influenced by both varietal and environmental controls or

limitations (Nicolas et al., 1984, 1985; Gleadow et al., 1982; Singh and Jenner, 1984). Cell division in endosperms from the third floret of intact and degrained spikes appeared to differ more in rate than duration, with cell number appearing to reach a plateau around 40 d.p.a. for both grain types, possibly up to ten days after mean ploidy had reached a maximum. The endosperms of other cultivars grown under comparable temperature and light regimes underwent cell division until 24 d.p.a. (Chojecki et al., 1986a) or beyond 30 d.p.a. (Radley, 1978). Chojecki et al. (1986a) also found that cell division continued beyond the mean ploidy increase and observed that this must reflect continued production of 3 C nuclei by mitosis, balanced by continued endoreduplication, in the same ratio.

It is also apparent that when DNA synthesis ceases at 40 d.p.a. (Fig. 2.3.3.1), for C grains from both intact and degrained spikes, so does cell division and the ploidy status of the endosperm remains constant through to maturity.

2.4.6 Endosperm cell dry weight

Radley (1981) measured an increase in endosperm cell number of 65% at 30 d.p.a. after similar grain removal experiments using the cultivar Kleiber. Although this figure was not unlike the mean 53.4% increase seen for Timmo (from 40 to 70 d.p.a) her data differed in that the increased dry weight of this particular experiment was scarcely discernable, even up to 42 d.p.a. It would therefore appear that degraining resulted in a considerable decrease in

endosperm cell dry weight in the remaining third floret grains.

A similar but smaller trend was revealed after the grain removal experiments described here with a mean 19.6% decrease in cell dry weight being found in the C grains of degrained spikes (Fig. 2.3.7.1). Since starch comprises between 70-80% of the dry weight of the wheat grain (Jenner, 1982) it is probable that the cell starch content of these grains must have been limited in some way. A comparison of the dry weight and cell number curves indicates that this must have been due to the inability of these cells to maintain the same rates of starch synthesis. The following chapter investigates the starch content of endosperms in order to determine how the number and volume distributions of starch granules are affected by degraining.

CHAPTER 3

WHEAT ENDOSPERM DEVELOPMENT: NUMBER AND SIZE DISTRIBUTION OF STARCH GRANULES OF LARGE AND SMALL GRAINS OF THE SAME GENOTYPE

3.1 Introduction

The grain removal experiments of the previous chapter revealed that the mean 43.8% and 40.0% increase in fresh and dry grain weights, respectively, attained in ears where the basal florets had been sterilised, was associated with a 53.4% increase in endosperm cell number, rather than with an increase in cell size. In fact, results from previous degraining experiments indicated that the cell starch content of these larger grains may be diminished relative to the amount of nitrogen-containing compounds (Jenner, 1980; Radley and Thorne, 1981). In addition, estimates of the average cell dry weight indicated that the larger grains, from degrained spikes, probably contained either fewer and/or smaller starch granules (chapter 2; Bhullar and Jenner, 1985; Brooks *et al.*, 1982).

The purpose of the experiments detailed in this chapter was to determine whether the starch content per cell is significantly different in the enlarged grains of partially sterilised spikelets and, if so, how the difference is effected. Data for the starch granule number and volume distributions were obtained using a Coulter counter

(Brocklehurst and Evers, 1977; Chojecki et al., 1986b; Baruch et al., 1979; 1983; Evers and Lindley, 1977) which also enabled the pattern and rate and duration of starch granule synthesis to be determined. The number of A type granules per cell is discussed as regards the possible number of endosperm plastids during development and in relation to mean cell ploidy.

3.2 Materials and Methods

Endosperm starch granule preparation

Starch granules were prepared from enzyme-digested endosperms and granule number and size distribution were analysed using a Coulter counter (Chojecki *et al.*, 1986b). Grains were rehydrated from acetic acid : ethanol and endosperms excised as described previously (section 2.2.4). For C grains from intact and degrained spikes (from Batch 1) there were three replicate endosperms from both mainstems and tillers. For A grains (from Batch 3.2) there were four replicate endosperms in each category.

Each endosperm was shaken in 1 ml of 1% cellulose and 1% Macerozyme, pH 5 (section 2.2.7), at 40°C for 15 h; then centrifuged at 13,000 x g for 3 min and the starch granule pellet resuspended in 1 ml of Isoton II (azide free, Coulter Electronics, Harpenden, Herts.). Centrifugation and resuspension was repeated three times and the granules were finally resuspended in 23.5 ml of Isoton. Appropriate volumes were further diluted in 25 ml of Isoton to produce a suspension of 5,000-15,000 particles per 0.5 ml: above 20,000 the results become less reliable due to coincidence counts. Duplicate dilutions were analysed for each endosperm. Background counts were kept below 200.

Coulter counter analysis

Numbers and sizes of endosperm starch granules were analysed using either a Coulter Counter Model TA (Batch 1 C

grains) or a Model TAII (Batch 3.2 A grains) (Coulter Electronics), both with a 100 μm diameter probe aperture. Before analysing samples, the instrument was calibrated using a suspension of latex particles which had a mean particle diameter of either 15.1 μm (Batch 1 C grains) or 19.5 μm (Batch 3.2 A grains). Results were presented as the total volume of particles in each of 15 size channels. Three counts of the number of particles in a preset volume of 0.5 ml were also recorded, the sample being inverted frequently before sampling.

Results for C grain (Batch 1) and A grain (Batch 3.2) endosperms were calibrated slightly differently. The former were analysed using size channels with mean particle volumes ranging from 2.57 μm^3 to 20398 μm^3 (Table 2.1). Data recorded were processed using a CBM Model 4032 microcomputer and Acucomp program (Coulter Electronics) to give the volume percentage of A type granules, total starch volume (in μl), modal volume of A types, as well as the volume and number percentage in each channel. For Batch 3.2 the Coulter counter was interfaced to an Apple II e microcomputer: using size channels ranging from 1.50 μm^3 to 49515 μm^3 (Table 3.1), figures were produced for the volume percentage of A type granules, modal volume of A types and volume and number percentage in each channel.

Table 3.2 Coulter counter mean channel volumes

Channel number	Mean channel volume in μm^3	
	Coulter TA	Coulter TAII
1	1.29*	1.50*
2	2.57	3.00
3	5.41	6.04
4	10.3	12.1
5	20.6	24.2
6	40.2	48.3
7	80.2	96.4
8	161	193.0
9	321	386.8
10	650	773.7
11	1288	1547
12	2572	3094
13	5204	6189
14	10421	12378
15	20398	24756
16	41630**	49515**

*Counts in channel 1 were not registered by the Coulter counter

** Counts in channel 16 were not included since they were more likely to represent debris or bubbles than starch granules.

3.3 Results

3.3.1 Starch granule number and volume during development

(a) Total number of starch granules

The curves for starch granule number per endosperm for C grains (from Batch 1) are sigmoidal: the most rapid increase spanning 35 to 50 d.p.a. (Fig. 3.3.1.1). It appears that starch granules were still being initiated up to 60 d.p.a., possibly even later. Thus starch synthesis may continue for a short time after the onset of grain dehydration. At 50 d.p.a. the mean total for C grain endosperms from degrained spikes was 35.7±25.3% greater than the mean for intact spikes, although the mean difference from 50 to 70 d.p.a. was only an increase of 33.7%.

A grains (from Batch 3.2) exhibited a similar rapid increase in endosperm starch granule number, from 23.3 to 27.4 d.p.a., (40-50 to 60-70 mg) after a long lag phase from 5.7 to 18.7 d.p.a. (0-10 to 30-40 mg) (Fig. 3.3.1.2).

(b) Starch granule size distribution

The total number of particles in each size channel was determined from the percentage of the total volume in each channel, as recorded by the Coulter counter, and the mean channel volume. Particle volume was determined from the amplitude of the change in electrolyte conductivity between two electrodes; therefore volume measurements of starch granules, which are reasonably spherical (see Fig. 3.3), would be expected to be quite accurate.

Histograms of starch granule size distributions show the

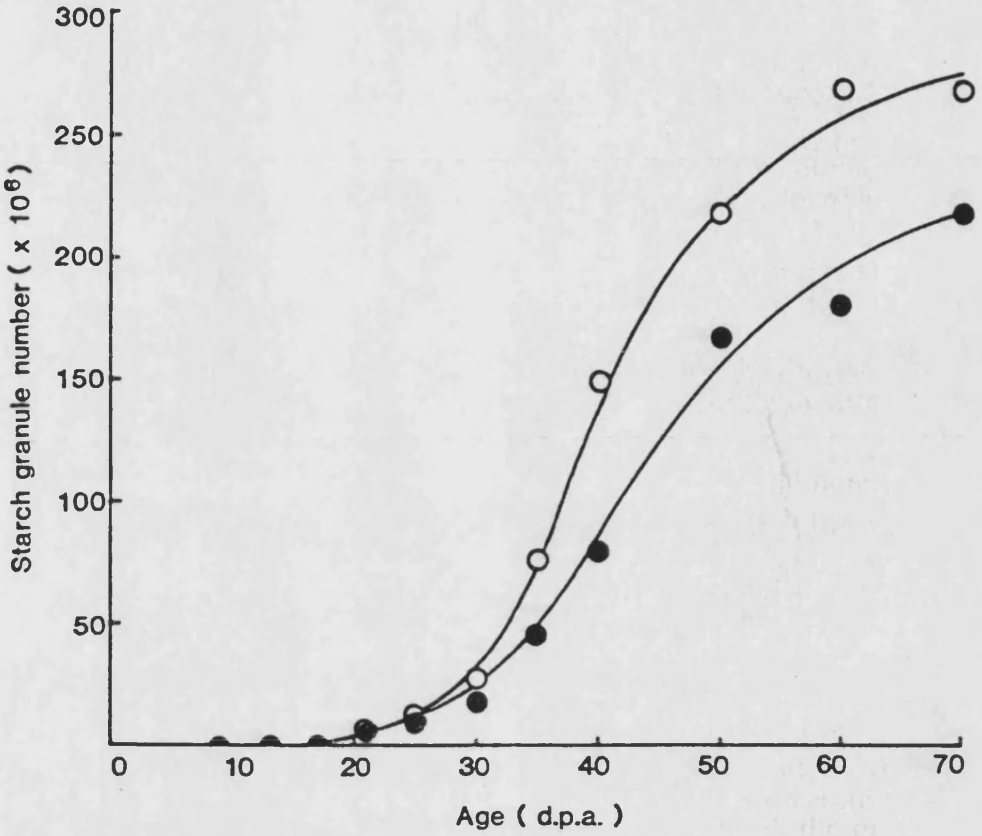


Figure 3.3.1.1 Total number of starch granules in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1)
Means were calculated from six replicate grains from three mainstems and three tillers.

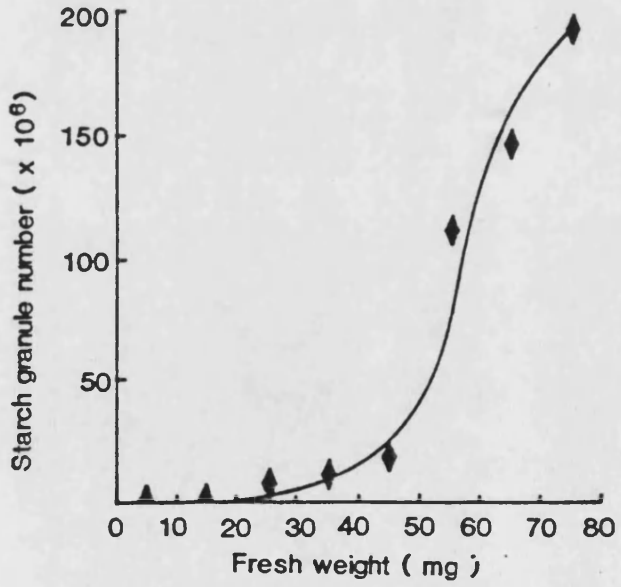


Figure 3.3.1.2 Total number of starch granules in A grain endosperms (from Batch 3.2)
Means were calculated from four replicate grains.

percentage of granules in each of fourteen predetermined size channels (Table 2.1). For C grains, representative data were selected showing the distribution at 17, 25, 35 and 70 d.p.a. Up till 17 d.p.a. as many as 77% of the starch granules in endosperms from intact spikes, and 65% from degrained spikes, fell in the two smallest size channels (average volumes 2.57 and 5.41 μm^3)(Fig. 3.3.1.3). By 25 d.p.a. starch granule volume had started to increase and as few as 40% were found in these channels; but from 35 to 70 d.p.a., as the endosperms matured, this percentage increased again as many small starch granules were initiated. At 70 d.p.a. the percentage of granules in the largest channels had fallen by comparison with endosperms at 25 and 35 d.p.a. and the smaller granules had increased in size.

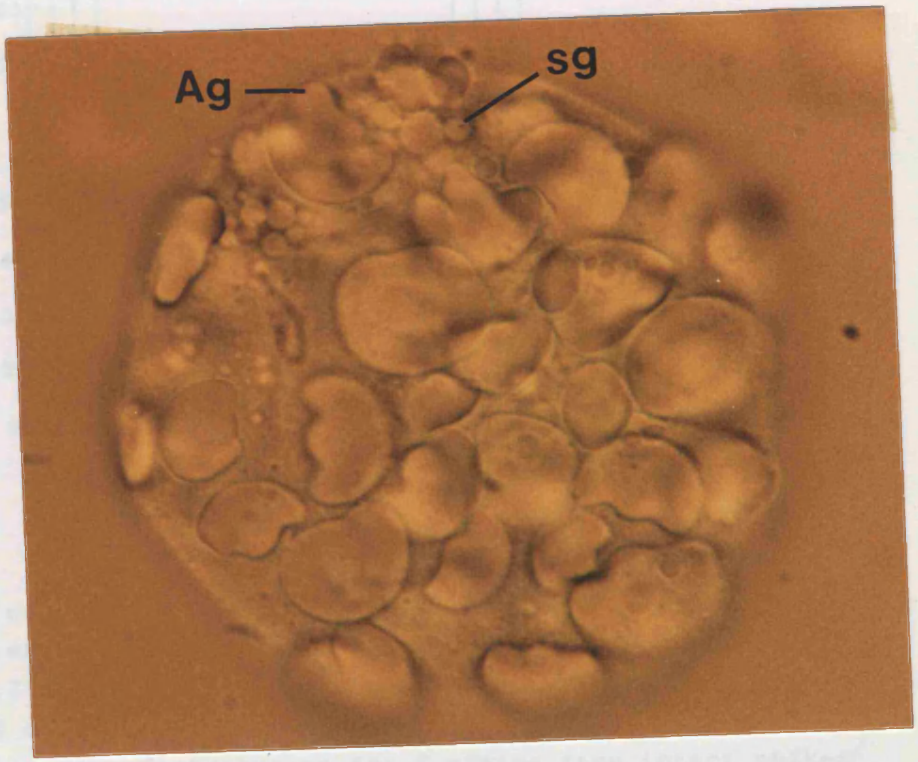


Figure 3.3 An endosperm protoplast at 14 d.p.a. under tungsten illumination
(magnification 40.8 μm)

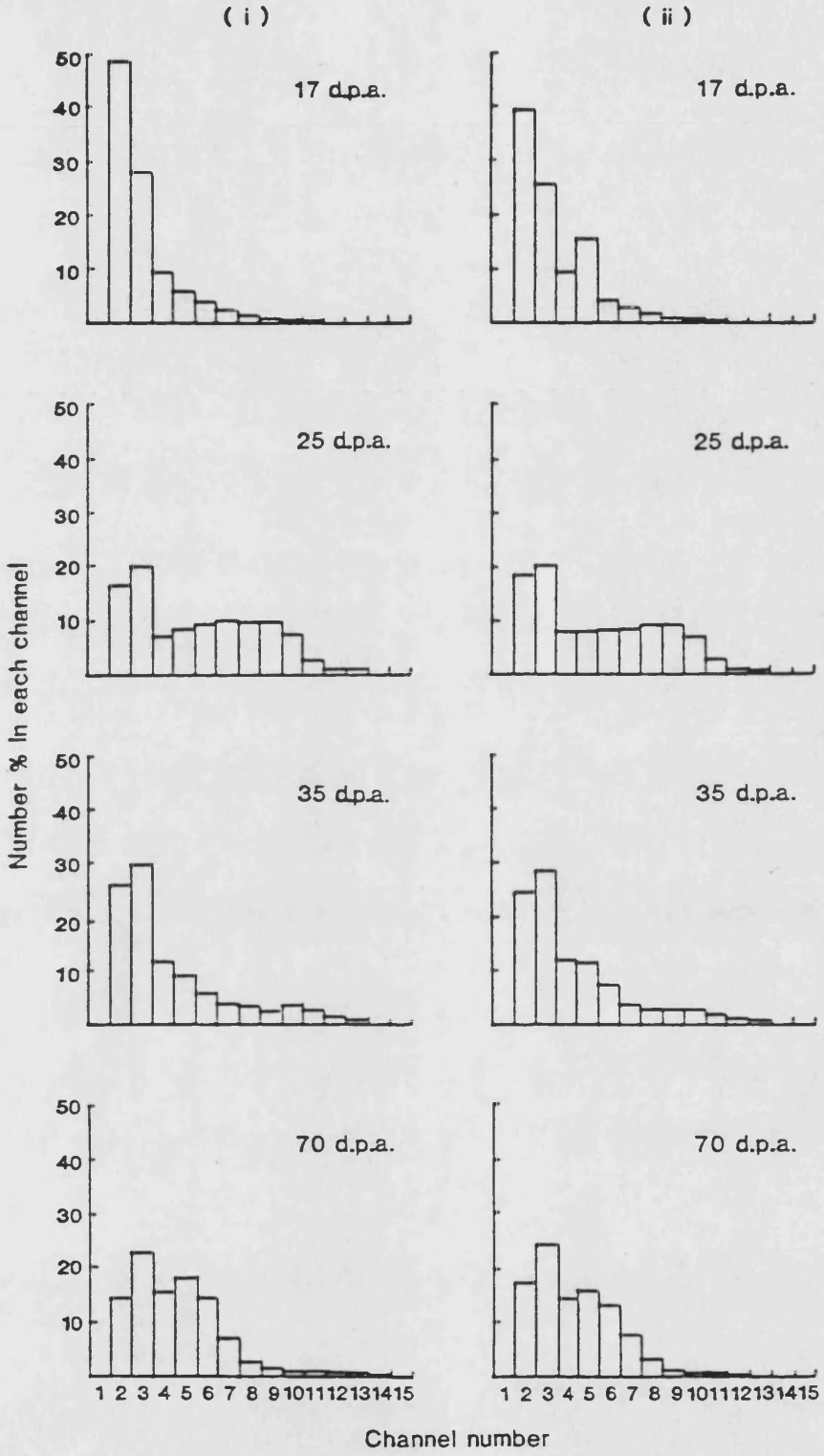


Figure 3.3.1.3 Histograms showing the percentage of starch granules in Coulter counter size channels 2-15 (see Table 3.2) for C grain endosperms (from Batch 1) at 17, 25, 35 and 70 d.p.a.:
(i) size distribution for C grains from intact spikes
(ii) size distribution for C grains from degrained spikes.
Means were calculated from six replicate grains from three mainstems and three tillers.

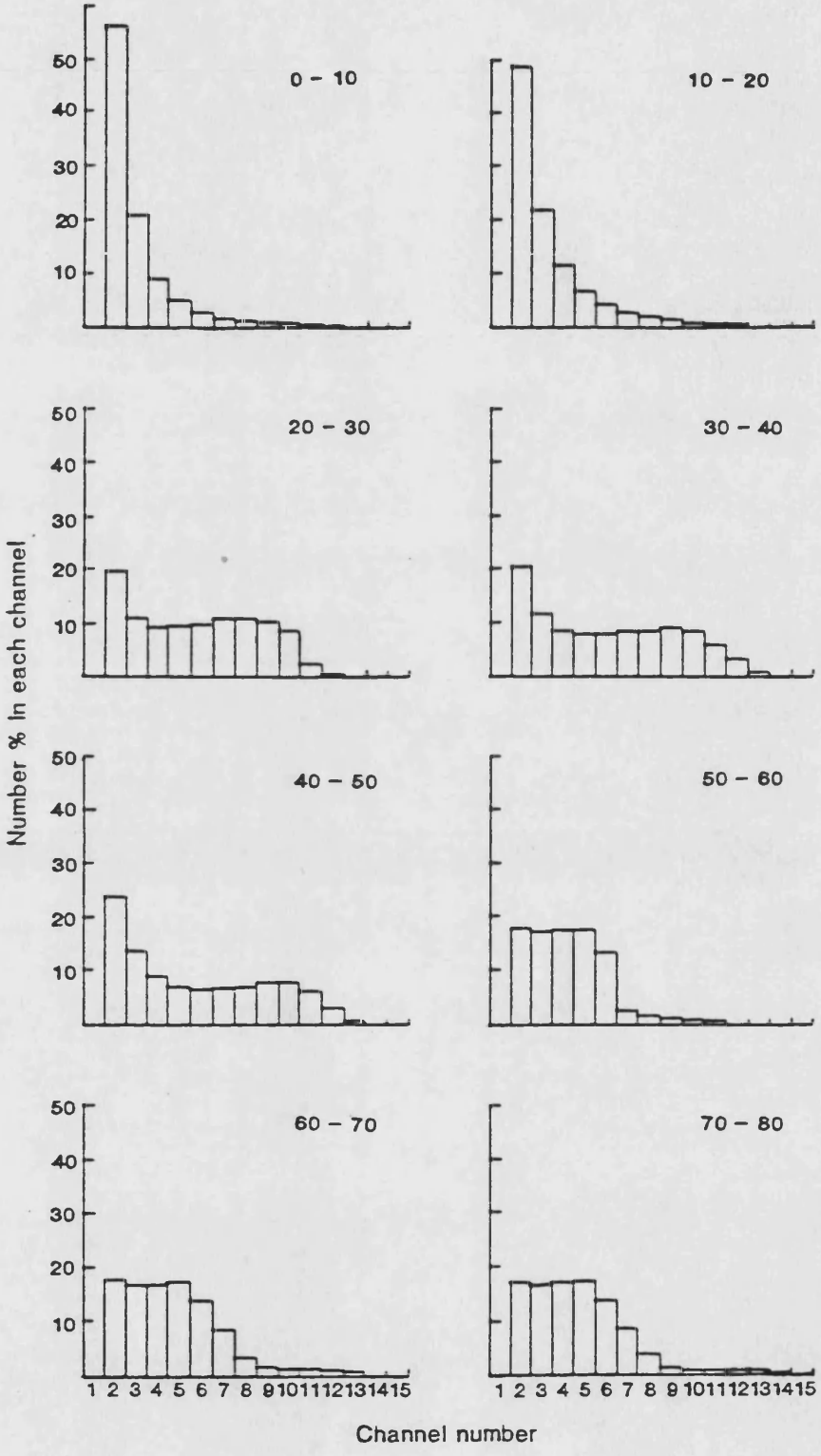


Figure 3.3.1.4 Histograms showing the percentage of starch granules in Coulter counter size channels 2-15 for A grain endosperms (from Batch 3.2)
Means were calculated from four replicate grains.

Histograms for endosperms from intact and degrained spikes, (Fig. 3.3.1.3(i) and (ii), respectively), showed very similar distributions with age.

The same pattern of development is apparent for A grain endosperms. Starch granule size increased considerably between 9.5 and 14.4 d.p.a. (10-20 and 20-30 mg, respectively), the distribution then remained fairly constant until 23.3 d.p.a. (40-50 mg), after which the percentage of small granules increased rapidly (Fig. 3.3.1.4).

(c) Starch granule volume distribution

The percentages of the total volume of starch granules in the same fourteen channels are presented in Figs. 3.3.1.5 and 3.3.1.6. For C grain endosperms at 17 d.p.a. the volume distribution appears to be quite constant with 3 to 15% being found in all of the channels. The volume percentages for channels 14 and 15 (average volumes 10421 and 20398 μm^3) may be artefacts caused by the proportionately larger numbers of air bubbles and debris in younger samples where granule number was quite low. By 25 d.p.a. a skewed distribution is evident, with the peak volume percentage being in channel 10 (average volume 650 μm^3). In maturing endosperms the distribution shifted as granule dimensions increased. Comparison of volume percentage and number percentage histograms for endosperms at 35 d.p.a., reveals two oppositely skewed histograms (Figs. 3.3.1.3 and 3.3.1.5); with a high volume percentage in larger channels constituting only a small percentage of total starch granule number.

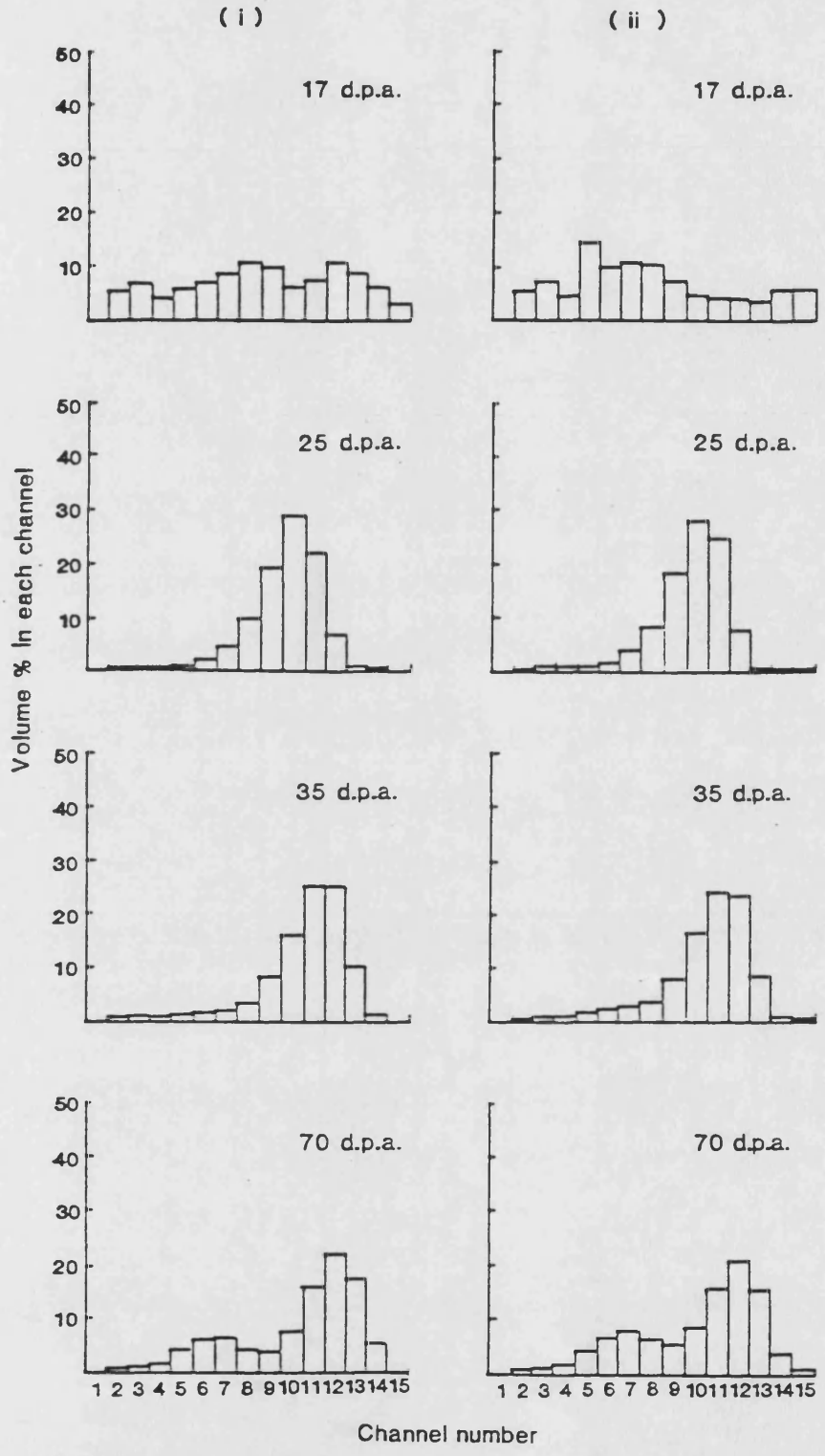


Figure 3.3.1.5 Histograms showing the percentage of the total starch volume in Coulter counter size channels 2-15 (see Table 3.2) for C grain endosperms (from Batch 1) at 17, 25, 35 and 70 d.p.a.:
(i) volume distribution for C grains from intact spikes
(ii) volume distribution for C grains from degrained spikes.
Means were calculated from six replicate grains from three mainstems and three tillers.

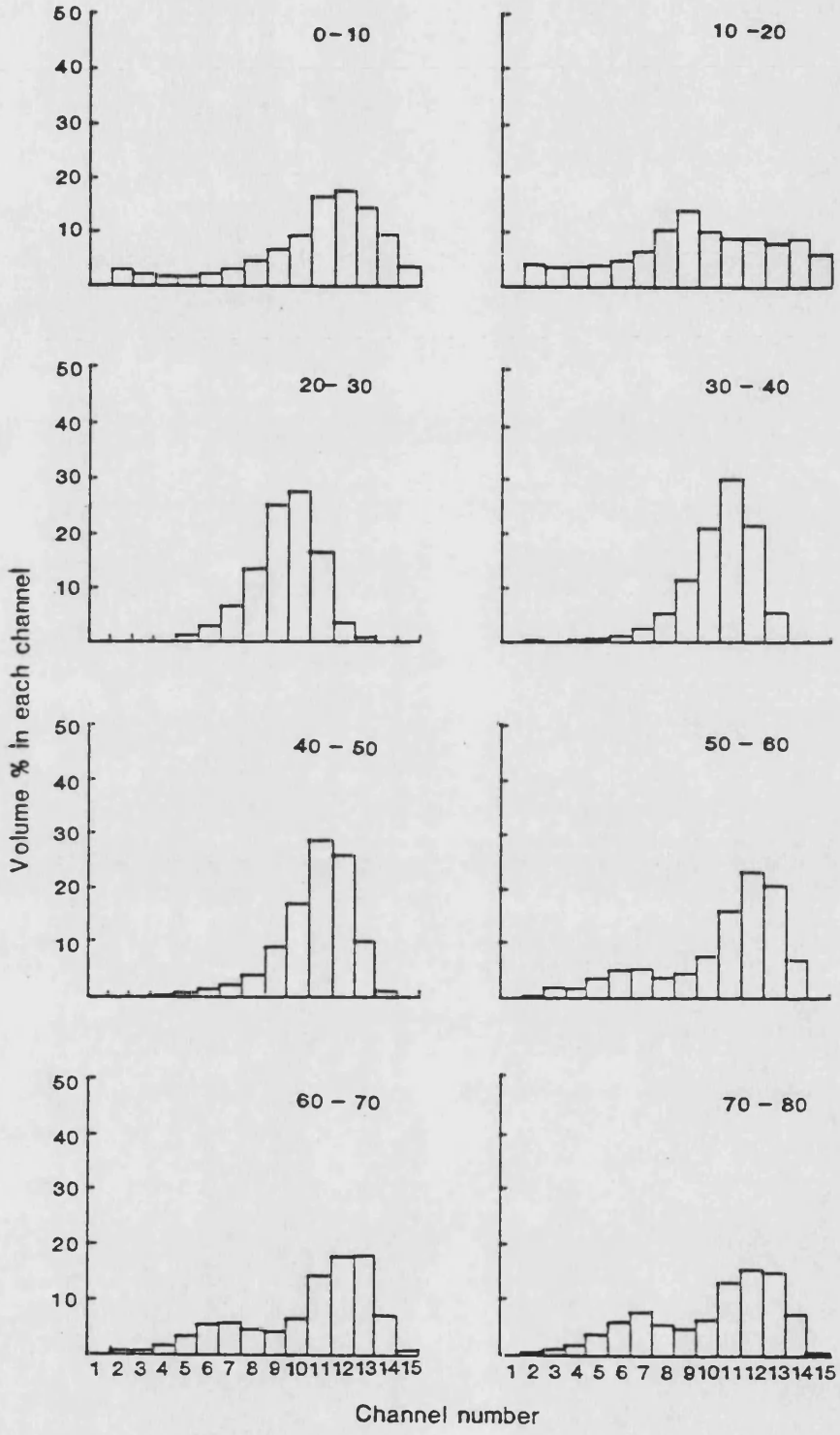


Figure 3.3.1.6 Histograms showing the percentage of total starch volume in Coulter counter size channels 2-15 for A grain endosperms (from Batch 3.2)
Means were calculated from four replicate grains.

From 50 to 70 d.p.a. a bimodal volume distribution is evident: the channel containing the lowest volume percentage, between the peaks for small and large starch granules, is channel 9 (average volume 321 μm^3). By 70 d.p.a. the volume percentage occupied by small granules, in channels 5 to 8 (average volumes 20.6 and 161 μm^3), had risen considerably due to B type granule initiation at around 35 d.p.a. Comparison of data for intact and degrained spikes shows little difference (Fig. 3.3.1.5(i) and (ii)).

For A grains the larger number of histograms demonstrates more clearly the transitions in endosperm starch granule volumes (Fig. 3.3.1.6). The same bimodal distribution, either side of channel 9 (average volume 386.8 μm^3), was evident from 25.7 d.p.a. (50-60 mg) onwards.

(d) Size transition and final number of A type starch granules

The lower limit of channel 9 was taken as the minimum size whereby the lenticular A type granules, which are initiated early on, may be differentiated from the smaller, spherical B types which appear later (Evers 1971, 1973). Therefore, only granules in channels 9 to 15 (average volumes 321 and 20398 μm^3 for C grains (Batch 1) and 386.8 and 24756 μm^3 for A grains (Batch 3.2)) are counted as A types. For C grains this corresponds to an approximate granule diameter of 8.5 μm or more. It was not possible to distinguish immature A type granules, occupying less than these volumes, from B type granules in channels 2 to 8 (average volumes 2.57 to 161

μm^3 for C grains and 3.00 to 193 μm^3 for A grains). The small discrepancy between the two grain types arose because the Coulter counters were calibrated slightly differently.

For C grain endosperms the number of A type granules in channels 9 to 15 rose rapidly from 21 to 40 d.p.a. (Fig. 3.3.1.7), approximately ten days prior to the rapid increase in total starch granule number. However, this is only an indication of the increasing dimensions of preexisting A type granules, not that A types are being initiated between 21 to 40 d.p.a. In fact, as described in section 3.3.2, it appears that few A types are likely to be initiated beyond 21 d.p.a. in C grains from either intact or degrained spikes, or beyond 14.4 d.p.a. in A grains (20-30 mg, Batch 3.2). The difference between numbers of mature A type granules in endosperms from intact and degrained spikes was similar to the difference in total granule number: from 50 to 70 d.p.a. the mean increase for degrained spikes was 28.3%.

A grain endosperms showed the same rapid increase in size of A type granules (Fig. 3.3.1.8) approximately five days prior to the large increase in total granule number.

(e) Modal volume of A type granules

Determination of the mean volume of A type granules was not possible as A type granules in channels below channel 9 were discounted. The closest approximation was to determine the modal volume of starch granules during development based on the percentage of the total volume in each channel. This was calculated from the peak channel (n) and two adjacent

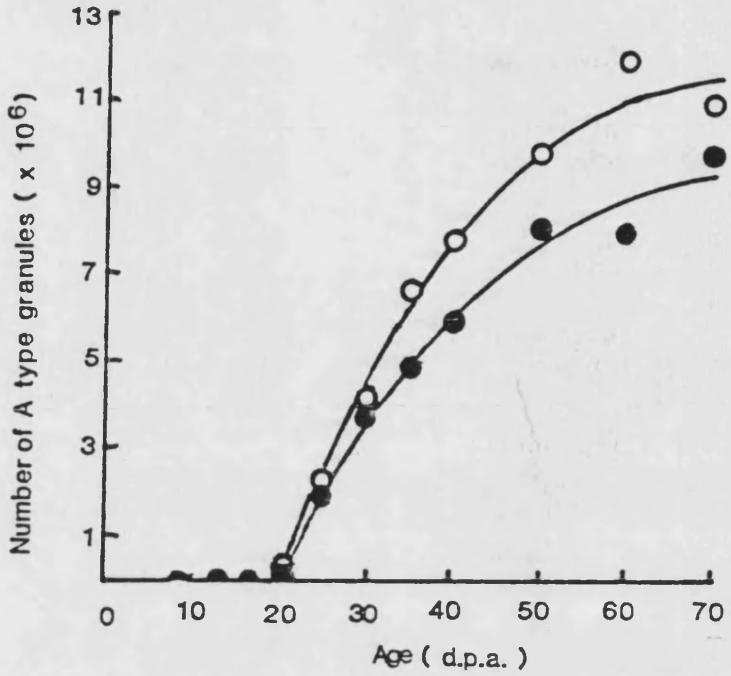


Figure 3.3.1.7 Total number of A type granules of more than 8.5 μm diameter in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1).

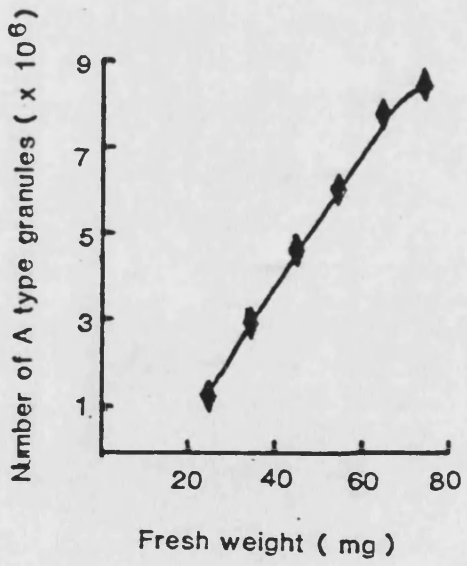


Figure 3.3.1.8 Total number of A type granules of more than approximately 8.5 μm diameter in A grain endosperms (from Batch 3.2)

channels on either side:

$$\text{Modal volume of A types} = \frac{\sum_{x=n-2 \text{ to } n+2} (\%\text{ total volume in channel } x)(\text{ x mean volume of channel } x)}{\sum_{x=n-2 \text{ to } n+2} \%\text{ total volume in channel } x}$$

The modal volume of A type granules in C grain endosperms increased from 110 μm^3 at 17 d.p.a. to 2954 μm^3 and 3197$\pm$305 μm^3 for intact and degrained spikes, respectively, at 60 d.p.a. (Fig. 3.3.1.9). The curves for endosperms from intact and degrained spikes indicated that A type starch granules not only developed to the same maximum size but also at a similar grain age; although the rate of development in endosperms from degrained spikes may have been slightly more rapid.

When the modal volume of A types from A grain endosperms was investigated, using a slightly different Coulter counter calibration (section 3.2) to that used for C grains and by Chojecki et al. (1986), a maximum modal volume of 4769 μm^3 was recorded (Figure 3.3.1.10, cf. Table 3.4). The fact that this value is greater than that for the C grains is due to the difference in the mean channel volumes used to estimate modal volume. It is thought that this, greater, figure may more accurately represent the true A type granule modal volume, since this accords better with stereological measurements of Timmo wheat granules (M. Bayliss, unpublished results).

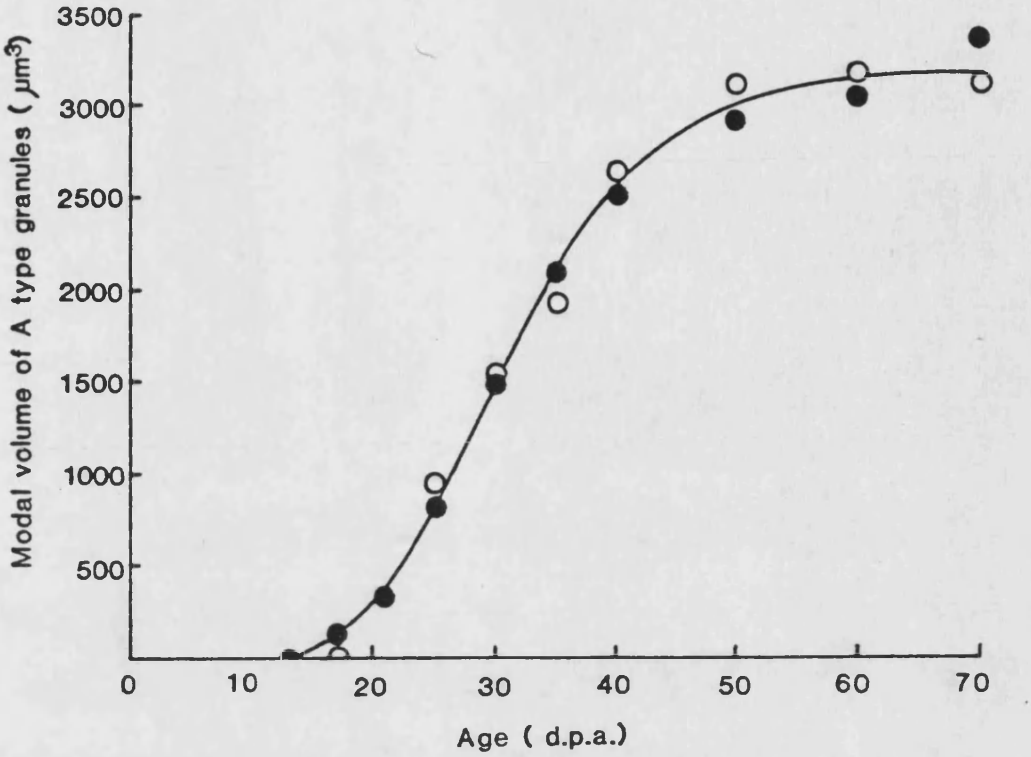


Figure 3.3.1.9 Modal volume of A type starch granules in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1)
Means were calculated from six replicate grains from three mainstems and three tillers.

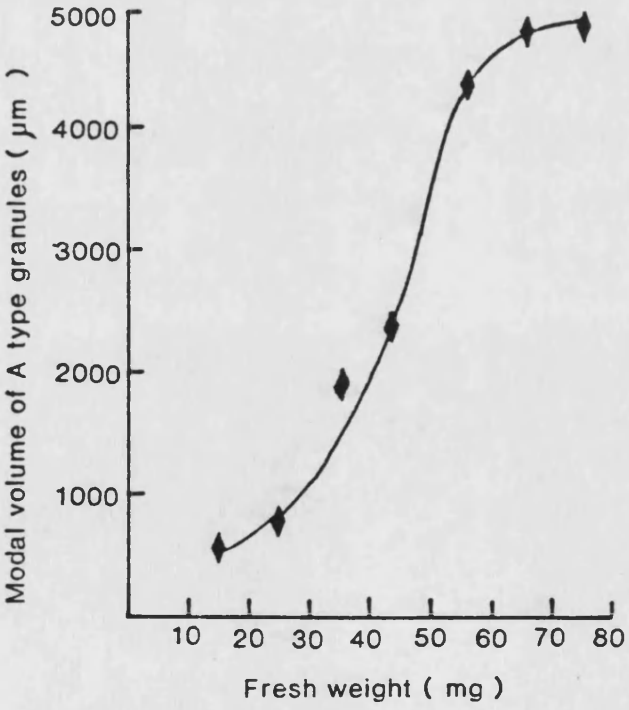


Figure 3.3.1.10 Modal volume of A type starch granules in A grain endosperms (from Batch 3.2).
Means were calculated from four replicate grains.

If the data for modal volume of A type granules is related to the estimated number of A types (channels 9 to 15), it is evident that for C grain endosperms up to 21 d.p.a. very few of the total number of A type granules are included in the estimated number, because the modal volume was less than 321 μm^3. Not until 30 d.p.a. are all of the starch granules that are included in the calculation of modal volume counted as being A type granules. The same is true for A grains less than 18.7 d.p.a. (30-40 mg).

(f) Total starch volume

The total volume of starch was calculated from the sum of the number of granules in each channel multipled by the corresponding average channel volumes. This was estimated for C grains only (Fig. 3.3.1.11). Total starch volume increased rapidly following the rapid increase in size of A type granules, slightly earlier than the rapid increase in total starch granule number, reflecting the significant contribution of A type granules to the total volume of starch. From 50 to 70 d.p.a. the mean increase in starch volume in C grain endosperms from degrained spikes relative to intact spikes was 34.5% (at 50 d.p.a. the difference was 44.0$\pm$19.7%). This was due to an increase in the number of starch granules rather than starch granule size (see (a) and (e)). It appears that starch volume, in parallel with the total number of starch granules, was still increasing after 50 d.p.a. (there was a significant increase in intact grains). This reflects the fact that B type granules are

initiated in quite mature endosperms: in addition, the modal volume of both A and B types may continue to increase beyond 50 d.p.a.

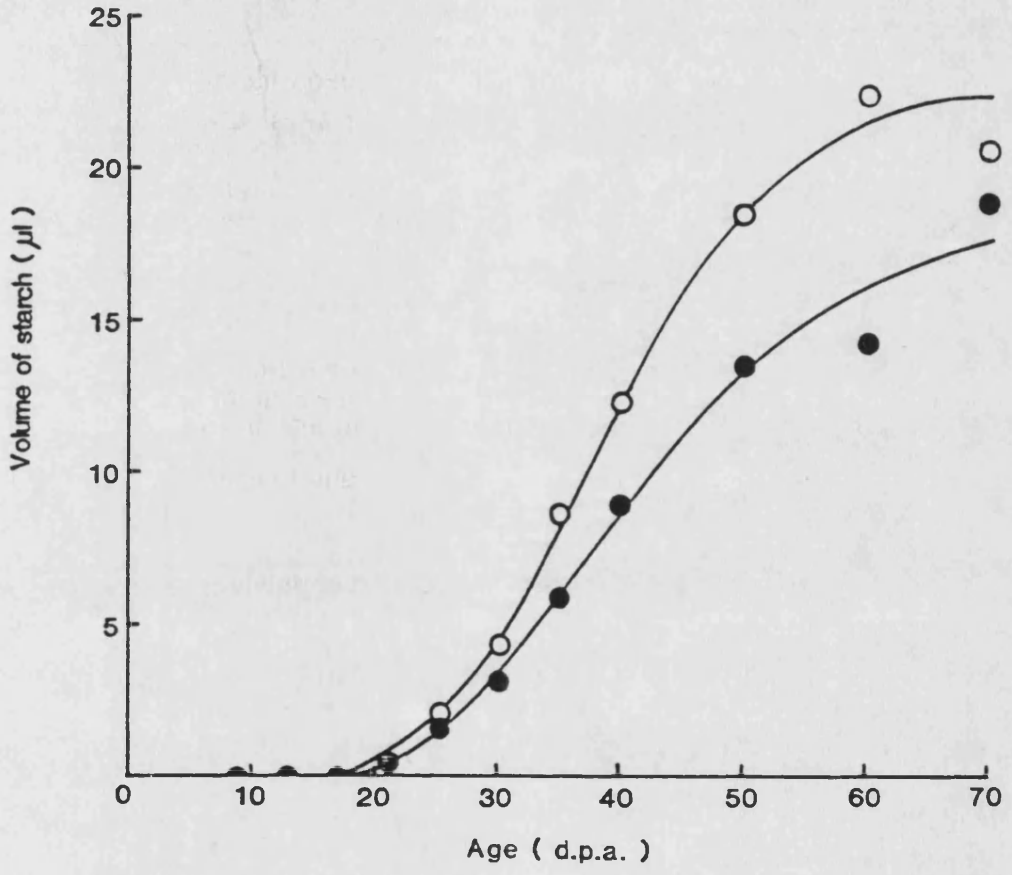


Figure 3.1.1.11 Total volume of starch in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1). Means were calculated from six replicate grains from three mainstems and three tillers.

(g) Percentage and volume percentage of A type granules

As discussed in section (d), numbers of A type granules in young endosperms are inevitably underestimated. However, for C grains, from 25 d.p.a. onwards, the data is probably reasonably representative, showing a rapid fall in the percentage of A type granules (from approximately 20%) to less than 4.5% by 50 d.p.a. (Fig. 3.3.1.12). It is conceivable that in the very immature endosperm 100% of the starch granules will ultimately develop into A type granules; the B types largely appearing from 30 to 50 d.p.a. From Figure 3.3.1.12 it would appear that the percentage of A types in endosperms from degrained spikes fell two to three days sooner than the percentage from intact spikes: that is, the increase in B type granules occurred earlier.

The percentage of A type granules in A grains fell extremely rapidly from 27.5% at 18.7 d.p.a. (30-40 mg) to 5.5% at 25.7 d.p.a. (50-60 mg) and decreased only slightly after this (Fig. 3.3.1.13). It was noticeable that development of A grains was, in all respects, considerably more rapid, showing sharper transitions than the C grains (from Batch 1). This must in part, be due to the higher greenhouse temperatures which enabled more rapid growth of the Batch 3.2 plants. The sharper transitions may be because A grains were harvested on a weight rather than an age basis, perhaps ensuring that endosperms of more comparable maturity were analysed together.

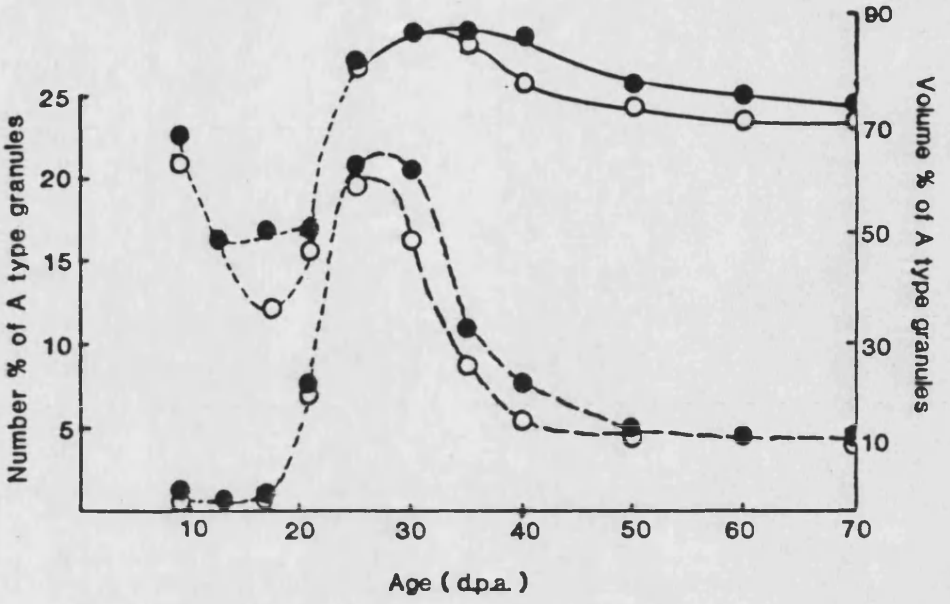


Figure 3.3.1.12 Percentage of starch granules that are A types (○—●) and percentage of total starch volume in A types (○—●) in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1).
(○---●) A type granules of less than 8.5 μm diameter are not included, therefore from 9 to 25 d.p.a. the percentages cannot be taken as representative.
Means were calculated from six replicate grains from three mainstems and three tillers.

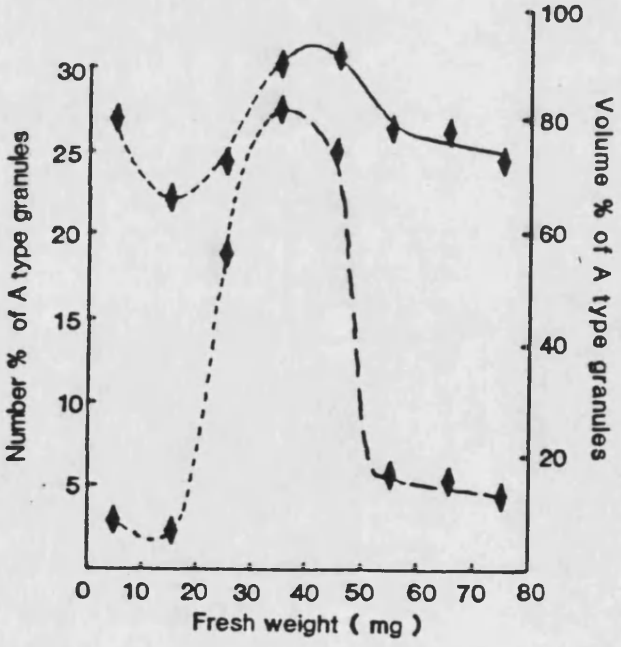


Figure 3.3.1.13 Percentage of starch granules that are A types (◆—◆) and percentage of total starch volume in A types (◆—◆) in A grain endosperms (from Batch 3.2)
(◆---◆) see legend for Fig. 3.3.1.11.
Means were calculated from four replicate grains.

Comparison of the volume percentage of A types with their percentage by number clearly shows how these granules, although relatively few in number, comprise a major proportion, at least 70%, of grain starch. As with the number percentage, the volume percentage of A type granules in endosperms from degrained spikes appears to fall several days earlier. In addition, the final volume percentage was 4.8% less than in endosperms from intact spikes but the difference is not significant.

3.3.2 Estimated starch granule number per cell

Endosperm cell number (Figs. 2.3.6.1 and 2.3.6.2), from mean ploidy and total DNA content, and starch granule data were used to estimate mean granule numbers per cell. Curves for total numbers per cell (Figs. 3.3.2.1 and 3.3.2.2) parallel those for total starch granule number (Figs. 2.3.1.1 and 2.3.1.2) with respect to a rapid increase from 30 to 60 d.p.a. for C grains (from Batch 1) and from 23.3 d.p.a. (40-50 mg) onwards for A grains (from Batch 3.2). A maximum of approximately 900 to 1000 granules per cell was attained in C grain endosperms from intact spikes.

Comparison of numbers per cell in endosperms from intact and degrained spikes suggests that these differ. For endosperms from degrained spikes at from 17 to 60 d.p.a., with the exception of 40 d.p.a., the number of granules per cell was approximately 16.7% less than for intact spikes. The mean difference from 50 to 70 d.p.a. was 17.6%, which was not significant. It appears that starch granule number per

cell was less in endosperms from degrained spikes except during the phase of rapid increase, possibly because these endosperms mature slightly earlier (as indicated in section 3.3.1(e)).

The numbers of A type granules per cell (Figs. 3.3.2.3 and 3.3.2.4) were not determined solely from the estimated numbers of A types (Figs. 3.3.1.7 and 3.3.1.8) because it was considered that, for C grains of less than 25 d.p.a. (Batch 1), or A grains of less than 18.7 d.p.a. (30-40 mg, Batch 3.2), these data are underestimated (see sections (d), (e) and (f)). It was therefore assumed that total starch granule number per endosperm (Figs. 3.3.3.1 and 3.3.1.2) more reasonably represents the true number of A types up to 21 d.p.a. (Batch 1) or 14.4 d.p.a. (20-30 mg, Batch 3.2). Thereafter, since the estimates of total number exceeded the estimated A type number at maturity, which were 8.98×10^6 and 11.58×10^6 for C grains from intact and degrained spikes, respectively, and 8.48×10^6 for A grains: these figures were taken as the A type granule number over the remaining period. This assumption was based on the knowledge that the B type granules were initiated later in endosperm development: from 25 to 70 d.p.a. (Batch 1) or from 18.7 d.p.a. onwards (30 to 40 mg, Batch 3.2). In addition previous reports have indicated that the early endosperm starch granules appear to develop into the larger A type granules, rather than being arrested at less than 8.5 μm diameter (May and Buttrose, 1959; Baruch et al., 1979; 1982; Chojecki et al, 1986b).

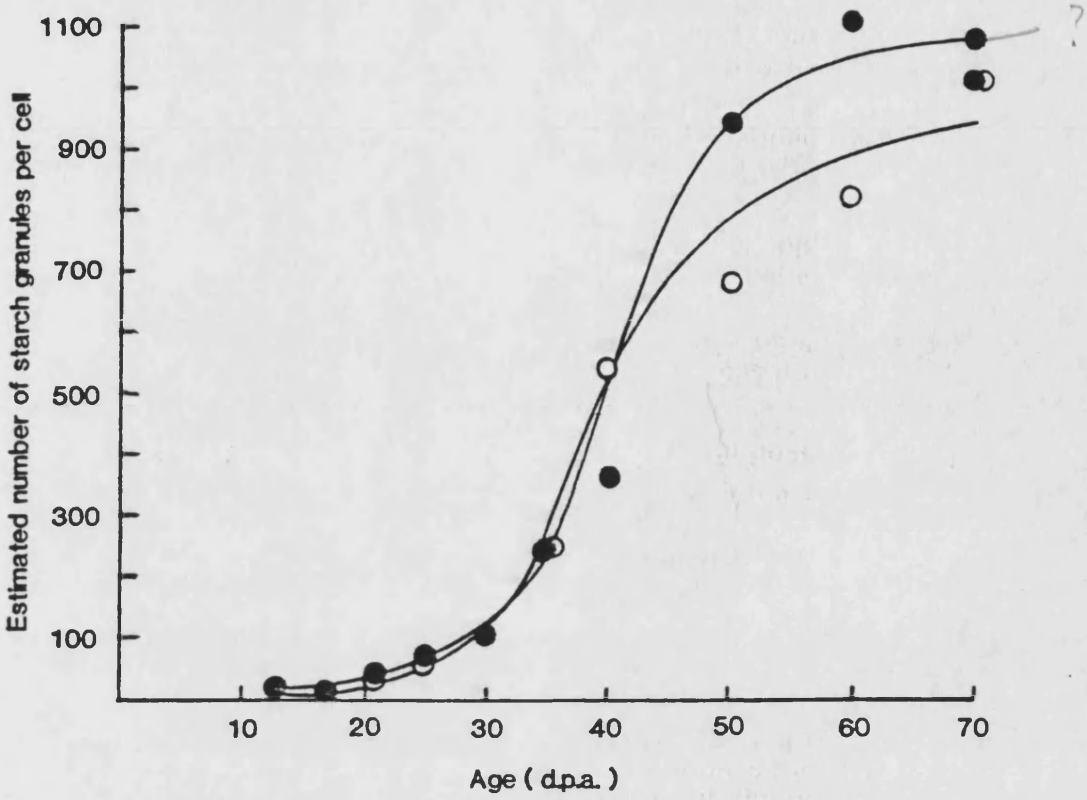


Figure 3.3.2.1 Estimated number of starch granules per cell in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1)

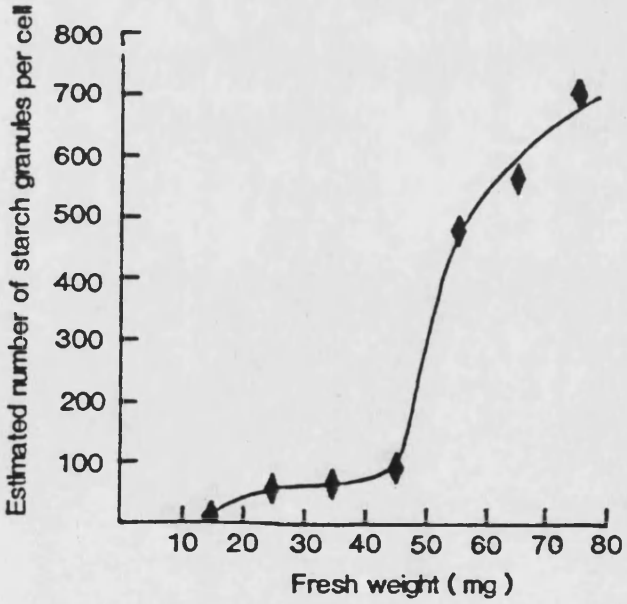


Figure 3.3.2.2 Estimated number of starch granules per cell in A grain endosperms (from Batch 3.2).

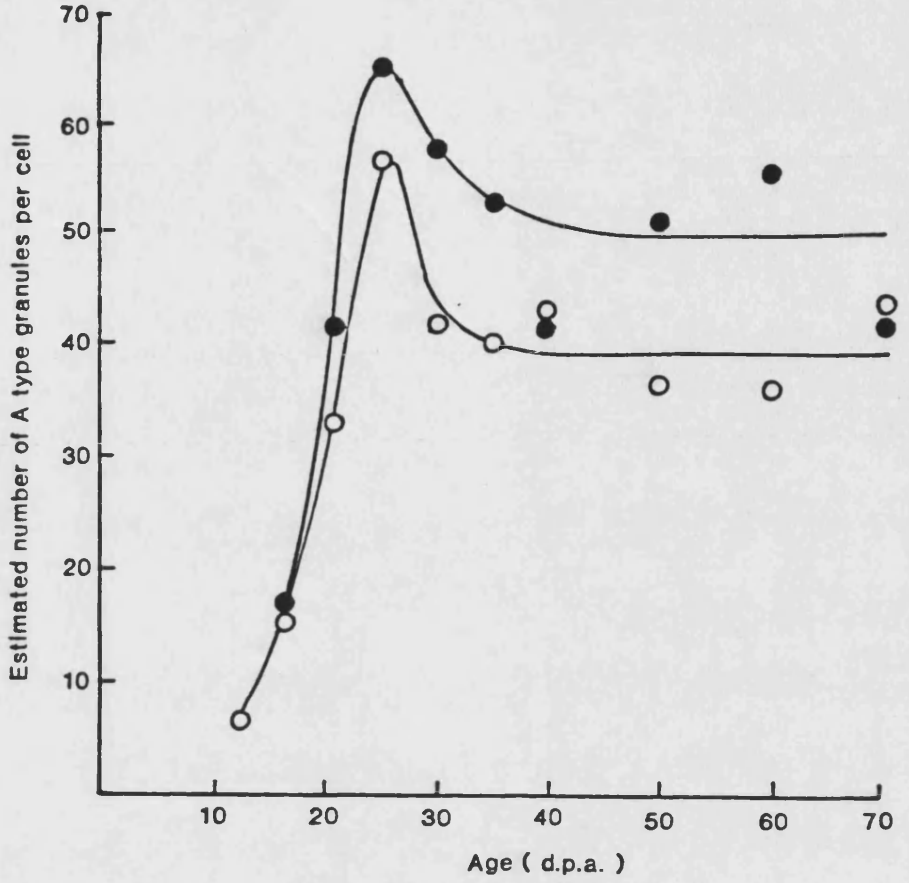


Figure 3.3.2.3 Estimated number of A type granules per cell in C grain endosperms from intact (●) and degrained (○) spikes (from Batch 1).

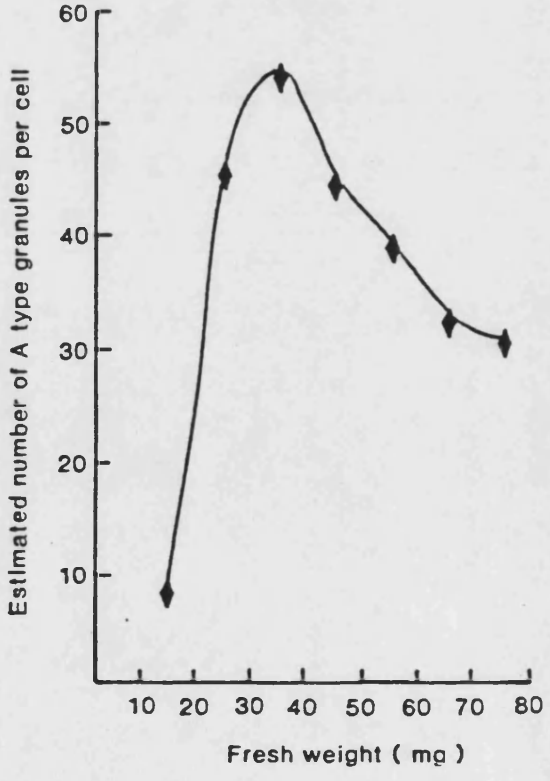


Figure 3.3.2.4 Estimated number of A type granules per cell in A grain endosperms (from Batch 3.2).

From Fig. 3.3.2.3 it is evident that the number of A type granules increased rapidly in the young endosperm, outstripping the rate of cell division, until a peak number of A types per cell was reached at 25 d.p.a.: numbers thereafter declined and then levelled at 30 to 35 d.p.a. It appears that the number of A type granules is delimited several days prior to the cessation of cell division, at around 40 d.p.a. (section 2.3.6).

A mean of 47.4±11.0 A types per cell was attained in endosperms from intact spikes over the period from 40 to 70 d.p.a., which is not significantly greater than the 39.9±6.5 A types per cell derived for endosperms from degrained spikes. However, from 25 to 70 d.p.a., there was a mean reduction of 16.9±14.7% for endosperms from degrained compared with intact spikes; a mean difference of 19.2% being recorded from 50 to 70 d.p.a. These figures are comparable to those for the reduction in total granule number per cell.

The trends for A grain starch granule number per cell (Fig. 3.3.2.2) are similar to those for C grains, as is the curve for the number of A types per cell (Fig. 3.3.2.4), with the exception that the latter fell from 54.5 for grains of 18.7 d.p.a. (30-40 mg) to 30.5 for grains of 70-80 mg, which is lower than the number for grains from Batch 1, at comparable developmental stages.

3.4 Discussion

3.4.1 Starch granule initiation and growth

A type granules

The initiation of starch granules in wheat and barley endosperm has been found to occur as early as 120 hours post anthesis, or sooner (Bennett et al., 1975; Evers, 1971; Williams and Duffus, 1977). The ensuing increase in starch granule number is considered to represent the increase in A type starch granules (May and Buttrose, 1959; Williams and Duffus, 1977; Briarty et al., 1979; Baruch et al., 1979). That these early starch granules are destined to enlarge as A types was supported by the volume percentage histograms (Figs. 3.3.1.5 and 3.3.1.6) which showed the size transition of the first granules initiated prior to the initiation of the smaller, B type granules. Since the total number of starch granules up to 21 d.p.a., for C grains from both intact and degrained spikes, was less than the final number of A type granules detected at maturity, and since the number of A types could not be directly determined, the total number figures were taken as being more representative of the true numbers over this period (section 3.3.2). From these data it appears that the A type granules were initiated until 25 d.p.a., with the most rapid increase in A type size occurring from 21 to 40 d.p.a. and smaller increases continuing through to maturation (Figs. 3.3.1.9 and 3.3.1.7).

It was also concluded that A type granule growth rates differed, either within the cells or across the endosperm.

This was because although the majority of A types were initiated in the 15 days prior to 21 d.p.a., when a number of granules had reached 320 μm^3 (Fig. 3.3.1.7) it took at least 20 more days for only two thirds of the total number to reach this size. A similar observation was made by Chojecki et al. (1986b); and Briarty et al. (1979) suggested that the rate of growth is possibly determined by the position of a granule within the endosperm. However, this is clearly subject to not only intrinsic (Bhullar and Jenner, 1985) but also environmental control (Brooks et al., 1982; Nicolas et al., 1984; 1985).

Williams and Duffus (1977) investigating immature barley endosperm found that starch synthesis was initiated at a number of sites within the amyloplast. Buttrose (1960, 1963) looking at wheat and barley found that usually single large granules developed in each amyloplast, and the B types were formed within membrane protusions or around the equator of the amyloplasts: this description was expanded by Parker (1985). Buttrose (1960) also reported that barley amyloplasts containing more than one starch granule underwent plastid division. Whether more than one granule may be initiated in wheat amyloplasts, as in barley, has not been reported (Bennett et al., 1975). It is therefore not possible to know whether early starch granule numbers are equivalent to the numbers of A types, since the initial starch deposits may coalesce (Williams and Duffus, 1977; Jenner, 1982). However, Evers (1971) looking at starch granule morphology of immature endosperms found that at as

early as 4 d.p.a. granules of less than 3 μm diameter resembled developing A types and these have not been shown to number more than one per plastid (Buttrose 1960, 1963). It is also not clear whether A type number is equivalent to plastid number in the young endosperm or whether a large number of young (pro) plastids are devoid of starch granules (Briarty et al., 1979; Kirk and Tilney-Bassett, 1978).

B type granules

After 21 d.p.a., for C grains from both intact and degrained spikes, most of the starch granules initiated were B types (Chojecki et al., 1986b). There was probably little overlap as regards the initiation of A and B types, as the majority of B types were not initiated until A type modal volume had exceeded 320 μm^3 (Figs. 3.3.1.1 and 3.3.1.3): in fact, the period of most rapid B type granule appearance (from 30 to 50 d.p.a.) coincided with the modal volume of A types approaching the maximum figure (Fig. 3.3.1.9). This fits with the pattern of granule development suggested by Williams and Duffus (1977) from ^{14}C-UDP glucose labelling experiments and with the appearance of a bimodal distribution of starch granule size (Fig. 3.3.1.4) (Chojecki et al., 1986b; Baruch et al., 1979; 1983). The initiation of B type starch granules continued until maturity (70 d.p.a.), when grain water content constituted only 45.1% of the grain dry weight (section 2.3.2).

Although it is not clear whether A and B type granules are of the same composition or whether the same enzymes are

involved in their synthesis (Parker, 1985), it seems likely that the B type granules are synthesized more slowly than the A types (May and Buttrose, 1959; Buttrose, 1960) and that, like the A types, on reaching a specific volume they are unable to grow further.

3.4.2 The ratio of A type to B type starch granules

Early reports on the size distribution of starch granules indicated that the B types constituted a relatively low fraction of the total number (Evers, 1973; Hughes and Briarty, 1976; Brocklehurst and Evers, 1977); however it is now apparent that A types usually constitute less than 10% of the total number at maturity (May and Buttrose, 1959; Chojecki *et al.*, 1986b). Under stress conditions, such as drought, the percentage has been found to decrease or increase, depending on when the stress was imposed (Table 3.4): results implied that the differences are caused by changes in the number of B types per cell, rather than A types (Buttrose, 1960; Bhullar and Jenner, 1985; Nicolas *et al.*, 1985; Brooks *et al.*, 1982), at least in mature grains (*cf.* Nicolas *et al.*, 1984).

In contrast, these grain removal experiments resulted in a comparable reduction in the number of both A and B types per cell, in endosperms of grains from degrained spikes (Figs. 3.3.2.1 and 3.3.2.3). Although endosperms from degrained spikes contained a slightly lower volume and number percentage of A type granules at maturity (70 d.p.a.), the difference between the two grain types was not significant

(section 3.3.1 (g)). Therefore, despite an approximate 17.6% reduction in the total number of starch granules per cell, as a consequence of grain removal, the number of B types produced per A type remained effectively constant.

3.4.3 Effects of degraining on A type modal volume and starch granule numbers per cell at maturity

Comparison of A type modal volume for C grains from Timmo wheat with those for Chinese Spring and Spica grains (Chojecki et al., 1986b) indicates that granule size is a cultivar specific trait (the same Coulter counter calibration was used in both instances, see section 3.2.1 and Chojecki et al., 1986b). The variation in average granule diameter for different cultivars had indicated this previously (Evers, 1971; Simmonds and O'Brien, 1981). Since A type granules comprise between 70 to 80% of the total volume of endosperm starch (Fig. 3.3.1.12) (Chojecki et al., 1986b) the average size of these granules at maturity has a significant effect on grain starch content. Therefore, stress conditions and seasonal variation, which have been found to limit A type granule development (Bhullar and Jenner, 1985; Brooks et al., 1982; Buttrose, 1960; Baruch et al., 1979), are likely to influence final grain yield. Despite the apparent variability of A type modal volume, at maturity, this did not appear to be sensitive to the removal of the basal grains (Fig. 3.3.1.9). It would appear that the final A type granule size, as well as the ratio of A to B type granules, was already maximal, for this genotype, in the endosperms

from intact spikes and therefore little affected by an enhanced assimilate and/or altered growth factor supply (Radley, 1978). This is in contrast to endosperm cell number which showed a mean 53.4±16.8% increase on grain removal (section 2.3.6).

In summary, degraining caused the cell starch content to be restricted and this was not effected by a reduction in granule size, within the starch containing plastids, but by a reduction in the number of both A and B type granules per cell (19.2% for A types and 17.6% for all starch granules, from 50 to 70 d.p.a.). A comparison of the data from other reports (Table 3.4) revealed that the estimated number of starch granules per cell and percentage A type granules at maturity were much more consistent, both between cultivars and between different treatments, than was the number of cells per endosperm. The effects of grain removal fitted in with this pattern, with the number of cells per endosperm being considerably more affected than the number of starch granules per cell. As discussed previously (section 1.3(iii)) endosperm cell division is responsive to factors extrinsic to the grain such as assimilate supply (Nicolas et al., 1984; 1985; Singh and Jenner, 1984) and growth factors (Radley, 1976; 1978; Singh and Jenner, 1982b) and, possibly, phloem unloading (Jenner, 1976; 1985a,b,c). In contrast, grain dry matter accumulation has been found to be less sensitive, most reports indicating a quite restrictive intrinsic control (Jenner and Rathjen, 1972a,b), especially as regards starch synthesis (Jenner and Rathjen, 1978) which

Table 3.4 A comparison of starch granule numbers and sizes in different wheat cultivars at the onset of maturation

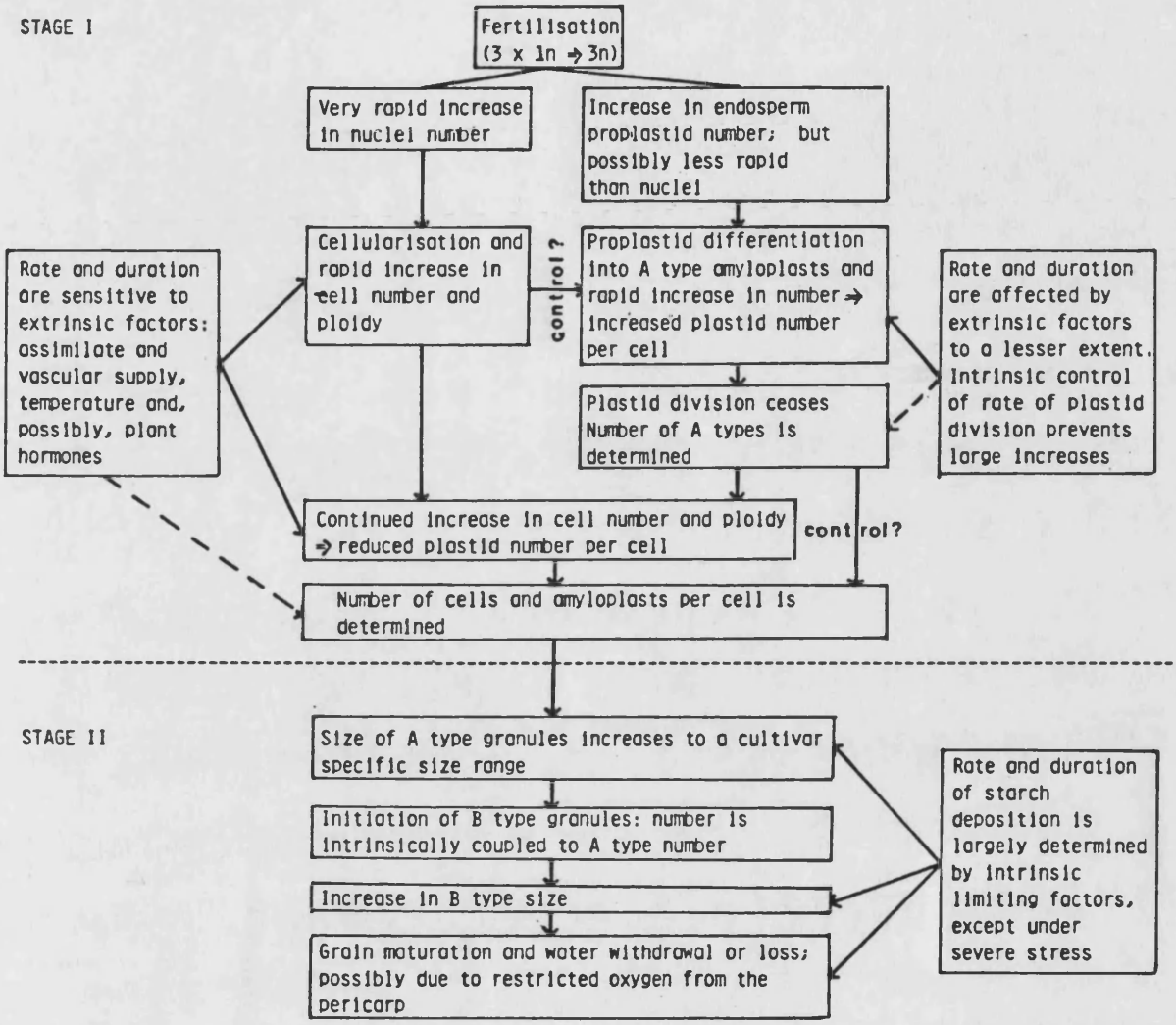
Wheat cultivar	Treatment	Percentage A types at maturity	Number of A types per cell	A type granule modal volume (μm^3)	Number of cells per endosperm	Number of granules per cell	Reference
Timmo (at 60 and 70 d.p.a.)	Intact spikes	4.47	47.5	3216	188700	1057	This thesis
	Degrained spikes	4.33	39.9	3150	291700	917	
Najah	none	6.71	-	-	-	1088	Gleadow et al. (1982)
WW15		5.81				1191	
Condor		4.86				1297	from Chojecki et al. (1986b)
T. sphaerococcum		7.24				688	
Kogat		3.97				755	
Chinese Spring	none	5.52	45.8	2535	240000	1001	Chojecki et al. (1986a,b)
Spica		4.86	55.7	3799	280000	1194	
Warigal	Control	3.70	40.0	-	59000	1137	Nicolas et al. (1985)
	Drought from 0 to 20 d.p.a.	6.06	37.0		51000	660	
Sun 9E	Control	6.28	-	-	-	-	Brooks et al. (1982)
	Drought from 6 d.p.a.	9.54					
Kolibri	none	5.00-4.36	50.0-41.0	-	105000	950-941	Briarty and Hughes (1979)

may ultimately be limited by the availability of oxygen from the pericarp (Gifford and Bremner, 1981). The results of the grain removal experiments reported in this thesis were in accordance with this description of the controlling factors and components of grain yield potential. In addition, the degraining experiments of Jenner (1980) where ears were reduced to four spikelets at 10 d.p.a. and no yield improvement was found also fit with this pattern as cell number and A type granule number were probably largely delimited prior to any changes in extrinsic factors being imposed on the developing grains. It is on the basis of these results and other reports (as discussed in section 1.3) that a model to describe how grain size may be determined was proposed (Fig. 3.4).

3.4.4 Effect of degraining on the rate of starch granule development

Although the mature size of A type granules did not differ significantly between the two grain types, from intact and degrained spikes (Fig. 3.3.1.9), it appears that the A types in endosperms from degrained spikes may have developed slightly more rapidly. The result of this was that the B type granules were initiated several days sooner in these grains, as reflected by the more rapid decline in the number and volume percentages of A types (Fig. 3.3.1.12).

It is possible, therefore, that the rate of starch synthesis per cell may be approximately equal in cells from the two grain types due to the more rapid rate of growth of a



STAGE I: Factors both extrinsic and intrinsic to the grain affect development

STAGE II: Intrinsic controls are more likely to be limiting development

Figure 3.4 A model of how wheat grain size is determined

17.6% smaller number of granules in cells of endosperms from degrained spikes. But when a critical A type granule size and predetermined ratio of A to B types are attained, starch synthesis ceases. However, that the rate of starch granule growth might vary between two grains of the same genotype is not supported by the experiments of Shannon (1974) who reported that the rate of ^{14}C incorporation into starch was positively related to granule surface area. As a result of this it was suggested that granule number and size may determine the rate of starch synthesis (Chojecki et al., 1986b).

3.4.5 Amyloplast number per cell throughout endosperm development

Although it is apparent that maturing A type granules are enclosed singly within the double membranes of a plastid, the amyloplast, it is not so clear whether the B types may be similarly enclosed (Buttrose, 1960; Parker, 1985). Results of electron microscopy indicate that B types are rarely independent of an A type, at least during the period of their synthesis (Parker, 1985). There is also no evidence for the presence of endosperm chloroplasts or plastids which do not contain A type granules (Parker, 1985), except during the first few days of development when proplastids are present prior to their transition to amyloplasts (Buttrose, 1960) (see section 3.4.1 as regards the initiation of A type granules). For these reasons the data presented for A type granule number probably reasonably represents the number of 'A type amyloplasts', but may not reflect the total number of

plastids, particularly in the youngest and the most mature endosperms.

A type amyloplast number per cell (Fig. 3.3.2.3) rose rapidly during the period of A type granule initiation, peaked, and then fell to approximately two thirds of the maximum, reaching a plateau when cell division ceased (Fig. 2.3.6.1). These trends reflect the cessation of A type synthesis prior to the end of cell division, therefore, since only the outer aleurone layer is meristematic (Sandstedt, 1946; Simmonds and O'Brien, 1981) it would appear that there is a decrease in the number of A type amyloplasts per cell towards the endosperm perifery. This is in accordance with the observation of Evers (1970) that the outer cells contain more protein and fewer granules.

Briarty et al. (1979) using stereological analysis revealed a similar trend, with the number of A types being determined prior to the cessation of cell division. They estimated that A type starch granules per cell increased from 16 to 148 and then fell to 50, in contrast to peaks of 65.0 and 56.5 and plateau figures of 47.4 and 39.9 for intact and degrained spikes, respectively. Their 'corrected' data for the number of A type amyloplasts, as opposed to granules, gave considerably higher values, with a much earlier maximum of 500 per cell, falling to a constant figure of 110 per cell. They suggest their discrepancy may be due to their failure to recognise small starch granules by light microscopy, so that A type granules were underestimated. Conceivably, a similar error may have caused A type

amyloplast number per cell to be underestimated in this thesis, as previously suggested (section 3.4.1). However, a figure of 500 plastids per cell in a tissue undergoing rapid cell division appears quite improbable, whereas the lower estimates recorded here accord better with other reports of plastid number per cell. Twelve has been cited as being a common plastid number in meristematic cells (Thomas and Rose, 1983) and young spinach leaf discs contain around ten, in mitotic cells, to up to 500 in fully differentiated cells (Possingham and Smith, 1972; Steele Scott and Possingham, 1983). Other reports investigating wheat and pea leaf mesophyll cells reveal increases in chloroplast number per cell from 24 to 240 (Ellis et al., 1983; Ellis and Leech, 1985; Dean and Leech, 1982; Lamppa et al., 1980).

3.4.6 Effects of degraining on amyloplast and cell division

The percentage difference between the number of A type amyloplasts per cell (and therefore also of total starch granules per cell) for endosperms from intact and degrained spikes increased from 9 and 13 d.p.a., where there was no significant difference ($P \leq 0.05$), to 21 d.p.a. where a 20.8% lower figure for endosperms from degrained spikes was recorded. (Thereafter, from 25 to 70 d.p.a. a mean 16.9 ± 14.7% lower number of A types per cell was recorded for endosperms from degrained spikes). The difference between the numbers per cell for the two grain types were, therefore, determined prior to the end of A type granule initiation (probably at 15 to 21 d.p.a.), before the cessation of cell

division (at 40 d.p.a.) and at least 40 d.p.a. prior to the end of B type initiation. From this it would appear that the rate of plastid division, or rather A type initiation, in the endosperms from degrained spikes failed to increase to the same extent as did cell division. Subsequently, after the number of A type amyloplasts had been determined, the same percentage difference, between the two grain types, was maintained due to approximately the same proportion of cell divisions occurring in both. These deductions raise a number of questions, such as: why does degraining cause the rate of cell division to increase more than the rate of plastid division, what controls the duration of plastid division and can plastid number ever be a determinant of cell number?

3.4.7 Relationship between cell ploidy and plastid number and volume

Butterfass (1973, 1983) proposed that plastid number per cell is controlled by the amount of nuclear DNA, since a pattern is frequently seen whereby plastid number increases with DNA replication or endopolyploidy. However, there are many exceptions to this relationship: plastid number per cell has also been found to depend on cell type (Olszewscka *et al.*, 1983) or tissue or cell age (Steele Scott *et al.*, 1984) and differentiation (Lamppa *et al.*, 1980), light intensity (Possingham and Smith, 1972; Bennett and Radcliffe, 1975) and quality (Possingham, 1973, 1980) and tissue nutrient status (Steele Scott and Possingham, 1983). It was therefore suggested that in some instances plastid volume, rather than

number, may be determined by the cell DNA content (Herrmann and Possingham, 1980), therefore plants in poor light conditions contain cells with fewer, but larger chloroplasts. In accordance with this Olszewscka et al. (1983) concluded that 'although chloroplast number per cell is species and cell specific, some relationship exists between the plastid index, plastid growth and the occurrence of nuclear DNA endoreduplication, (but they found) no correlation between the level of nuclear endopolyploidy and the number of chloroplasts per cell'. In addition, Ellis et al. (1983) concluded that 'chloroplast replication is not dependent on nuclear endoreduplication in seedling leaves of wheat' and they later determined that chloroplast number in fully expanded mesophyll cells was positively correlated with cell plan area, over a five-fold range of cell size in isogenic diploid and tetraploid T. monococcum (Ellis and Leech, 1985). For T. aestivum this relationship varied depending on the chloroplast plan area, thus chloroplast face area per unit cell plan area was similar in all cells. Since DNA content is strongly correlated with cell volume and nuclear volume, as well as with cell cycle length and minimum generation time (Smith, 1973; Cavalier-Smith, 1978), it would appear that cell area may more closely determine plastid number and volume than does nuclear DNA content or nucleus size. In this context it has been found that cells of a similar size, but from wheat species of different ploidies, have similar numbers of chloroplasts (Pyke and Leech, 1987).

In this thesis it was found that for cells of a similar

ploidy distribution (section 2.3.4), which were highly differentiated, that the number of A type amyloplasts per cell differed by 19.2% from 50 to 70 d.p.a. If the hypotheses stated above hold true for plastids other than chloroplasts it would be expected that either plastid size should be greater in endosperms from degrained spikes, which was not the case if starch granule size is an indication of amyloplast size (Fig. 3.3.1.9); or cell size, or rather plan area, should be less in these endosperms. In fact, it is very probable that cell size was reduced, as indicated by the estimated cell dry weights (section 2.3.7.1), but since starch, and therefore plastid content, is the major constituent of the wheat endosperm (Jenner, 1982) the argument is no longer rational. In addition, it is perhaps unlikely that the same intrinsic controls will directly determine the number and distribution of amyloplasts as have been suggested to determine either chloroplast number or volume per cell. The number and volume of chloroplasts per cell plan area, ~~as~~ but not opposed ~~to~~ volume, is clearly of critical importance as regards maximal light interception; whereas endosperm cell shape, and hence plan area, is more likely to be optimised for diffusion and transport of assimilate (Jenner, 1985a,b,c) and, possibly oxygen (Gifford and Bremner, 1981; Duffus, 1979).

Results discussed in section 3.4.4 indicated that the net rate of starch synthesis in cells from endosperms of degrained and intact spikes may be similar, despite differences in the number of granules per cell. In this

context, it has been suggested in a number of reports that the rate of starch synthesis may be limited by a nuclear encoded enzyme, such as sucrose synthase (MacDowell Date and Housley, 1986), ADP glucose pyrophosphorylases (Duffus, 1979) or starch synthase (Rijven, 1986) and that cell ploidy determines the cell complement of a particular enzyme (Dean and Leech, 1982b; Leech et al., 1985). As cell ploidy does not differ significantly between the two grain types (Fig. 2.3.4.1), it is conjectured that a comparable rate of synthesis of starch may also be the result of similar rates of synthesis of a particular enzyme.

CHAPTER 4

PLASTID DNA IN WHEAT ENDOSPERM

4.1 Introduction

The mature wheat endosperm has been found to comprise at least 8.5×10^6 A type amyloplasts and a total of 194×10^6 starch granules (this thesis sections 3.3.1(a) and (d)). The primary aim of this project was to investigate whether ptDNA is present in these amyloplasts and, if so, to determine whether plastome copy number per cell more closely parallels the increase in numbers of A or B type starch granules per cell or the endosperm mean cell ploidy (sections 3.3.2 and 2.3.4). The latter was found to increase from the first triploid fusion nucleus (Evers, 1974) to an average 5.25 C in A grains of 40-50 mg. It was also found that A type amyloplasts per cell reached a maximum of 54.4 and then fell to 30.5, in grains of 30-40 and 70-80 mg, respectively, whereas B type starch granules were not initiated until grains reached 50-60 mg, thereafter rapidly increasing in number to attain a mean 666 total starch granules per cell in grains of 70-80 mg.

The extraction of intact amyloplasts free from cellular debris was not well documented when this work was started (Lobov and Bondar, 1977; Bondar *et al.*, 1979; Liu and Shannon, 1981); although a number of more recent reports claim to have recovered reasonably pure and intact fractions from pea epicotyls (Gaynor and Galston, 1983) and roots (Emes

and England, 1986), cereal endosperm (Rijven, 1986; Echeverria et al., 1985) and soybean suspension cultures (MacDonald and ap Rees, 1983). It was therefore our intention to use wheat ctDNA as a probe for endosperm ptDNA in total endosperm DNA, and to quantitate the ptDNA by reassociation kinetics (Chelm, 1982; Britten et al., 1974; Britten and Kohne, 1968) and, later, by probing filter bound DNA using the dot blot technique (Kafatos et al., 1978). The presence of plastid nucleoids in DAPI-stained amyloplasts liberated from lysed endosperm protoplasts was also investigated.

4.2 Materials and Methods

Glassware and buffers

All glassware was silicon coated by rinsing in dimethyl-dichlorosilane (BDH Lab. Reagent), baking at 120°C, rinsing thoroughly in distilled water and drying. Polypropylene microfuge tubes, used for dilute DNA or RNA solutions, were also silicon coated. Buffers, distilled water and salt solutions were either autoclaved at 20 lb/in^3 for 20 min, or filter sterilised using 0.2 μm Acrodiscs (Gelman Sciences, Inc.). Buffers containing glucose or sucrose were autoclaved at 10 lb/in^3 for 15 min. Unless otherwise stated, all reagents came from Sigma Chemical Co.

4.2.1 Total DNA extraction from leaves and endosperm

The DNA extraction procedure was largely as described by Blin and Stafford (1976). CsCl density gradient centrifugation was included to separate RNA and DNA (Steele Scott and Possingham, 1980).

Total DNA was extracted from Batches 2, 3.1 and 4 endosperms that had been stored at -20°C: the grains from Batches 3.1 and 4 had been weighed and sorted into size categories, rather than age (sections 2.2.2 and 2.2.4). Total wheat leaf DNA was also extracted (cv Timmo). Leaves, measuring 30-40 cm in length, were harvested from $4\frac{1}{2}$ week old, growth cabinet grown plants. Endosperm tissue weighing 0.5-2.0 g was sufficient for each extraction.

Endosperms from Batch 2 and wheat leaf material were ground to a fine powder in liquid nitrogen, using a pestle

and mortar, and freeze dried in an Edwards Modolyo 8 two stage vacuum drier at 6-8 x 10^{-2} mbars, overnight. Excess freeze dried leaf material was stored over silica gel at -20°C, whereas endosperm material was extracted immediately. Freeze dried powders were reground, 0.1:4 w/v in Buffer T: 0.1M Tris-HCl (2-amino-2-(hydroxymethyl)propane-1,3-diol-HCl, SLR grade, Fisons), 5 mM EDTA (ethylenediaminetetraacetic acid, disodium salt), 1% w/v Sarcosyl(N-lauroylsarcosine), 0.1 mg/ml ethidium bromide (3,8;diamino-5-ethyl-6-phenyl-phenanthridium bromide) and 1 mg/l protease (from Streptomyces griseus, Type XIV), pH 8.0 at 4°C. Buffer T had been predigested at 37°C for 2 h, to eliminate DNase (deoxyribonuclease) activity. Ethidium bromide was included as a nuclease inhibitor (Kislev and Rubinstein, 1980). The endosperm and leaf homogenates were incubated at 37°C for 1 h, with frequent shaking.

It was considered that freeze drying could cause amyloplast DNA to become more closely associated with the starch granules, thereby affecting the relative extraction efficiencies of plastid and nuclear DNA. For this reason subsequent extractions, from Batch 3.1 and 4 endosperms, omitted the freeze drying step: tissue was ground in liquid nitrogen, thawed and reground in Buffer T and incubated at 37°C.

The homogenate was phenol extracted 1:1 v/v with water saturated phenol, which had been equilibrated at pH 8.0 using 10 x TE (TE is 10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Each phenol extraction was shaken for 10 min, centrifuged in a MSE

bench centrifuge at 2,300 x g, for 10 min, and the upper aqueous layer retained. The interface and lower layer from the first extraction were reextracted with 0.5 vol of TE and the two aqueous layers combined and phenol extracted two or three more times. The aqueous layer was then extracted three times with chloroform: isoamylalcohol (24:1 v/v), using the same procedure.

Caesium chloride density gradient centrifugation

Nucleic acid solutions were mixed 7:1 v/v with 8 x saline sodium citrate (SSC is 0.15M NaCl and 15 mM trisodium citrate, pH 7.0) and 10-20 μl of 10 mg/ml ethidium bromide was added to each 4.3 ml. CsCl (Rose Chemicals Ltd.) was added 1:1 v/v to obtain a refractive index of 1.396-1.400. The solution was heat sealed into Beckman 'quick-seal', polyallomer, 2 in tubes which were centrifuged in a Beckman Vti 65 vertical rotor in a Beckman L5-50B centrifuge at 10°C and 200000 x g, for 17 h. Horizontal bands of DNA were located under u.v. light from a transilluminator (Model TM20, U.V.P., Inc., Ca., U.S.A.) (Fig. 4.1) and recovered using a syringe with 21 or 26 gauge needle (Beckton and Dickinson and Co.) inserted into the tubes below the band.

To remove salt, the DNA was diluted with 3 vol of TE, centrifuged in a Centricon-10 microconcentrator (Amicon Corp., U.S.A.) at 5000 x g, 4°C, for 2 h, and the concentrate retrieved. Ethidium bromide was extracted from the DNA using three equal volumes of butan-1-ol: each time the immiscible layers were mixed thorougly and the lower, aqueous layer

retained.

DNA was precipitated by adding 0.1 vol of 3M sodium acetate, pH 5.5, and 2 vol of 95% ethanol: the solution was left either at -20°C, overnight or at -80°C for 30 min. Precipitated material was pelleted at 13000 x g for 15 min, dried under vacuum for 5 min, resuspended in TE and stored at -20°C.

Figure 4.2 CsCl gradients under ultra violet illumination showing horizontal bands of linear DNA.

4.2.2 Chloroplast DNA extraction

Chloroplast preparation

Wheat leaves (cv Timmo) were harvested from either growth cabinet or greenhouse grown plants, when 2-7 weeks old. Plants were usually 'destarched' in the dark, overnight. Chloroplasts were extracted in a cold room at 4°C by differential centrifugation (Dyer, 1982) and sucrose density gradient centrifugation (Rocha and Ting, 1970).

Leaves weighing 100-200 g, were washed in 0.1% w/v

Sarcosyl and rinsed in distilled water. They were homogenised 1:4 w/v in semi-frozen Medium C: 0.25 M Tris-HCl, 0.35 M sucrose, 3 mM EDTA, 0.04 M 2-mercaptoethanol (Aldrich Chem. Co.) and 0.05% bovine serum albumin (BSA), pH 8.0; for 3 x 5 s bursts in a Waring blender, on maximum. The homogenate was strained through four layers of sterile muslin and centrifuged at 200 x g for 2 min, to pellet cell debris. The supernatant was retained and centrifuged at 2,300 x g for 2 min, to pellet chloroplasts; the resultant supernatant was discarded and the pellet resuspended gently in a similar vol of Medium C, using sterile, non-absorbant cotton wool and a glass rod. Chloroplasts were repelleted, as before, resuspended in Medium C and layered onto discontinuous sucrose gradients in Beckman 50 ml SW 27 cellulose nitrate tubes. Gradients were prepared, by sequentially layering 7.5 ml of 55, 43, 32 and 20% w/v sucrose solutions in Buffer S: 10 mM Tris, 1 mM EDTA and 5 mM 2-mercaptoethanol, pH 8.0; and were left at 4°C overnight. They were centrifuged in a Beckman SW28 swing out rotor at 4°C, 91000 x g, for 35 min. Chloroplast bands were removed from the tubes, diluted 1:1 v/v with Medium C and centrifuged at 4°C, 8000 x g, for 20 min. The pellet was resuspended in TE containing 2-4% w/v Sarcosyl and frozen at -20°C to lyse chloroplasts.

DNA extraction

DNA was separated from RNA and protein by CsCl density gradient centrifugation (Kolodner and Tewari, 1975), as described in section 4.2.1. Wheat ctDNA banded in either one

or two horizontal bands; each was removed separately. The lower band was covalently closed circular ctDNA (ccctDNA) and the upper was both linear ctDNA and any nuclear DNA (nDNA) contamination (Kolodner and Tewari, 1975; Vedel et al., 1976).

4.2.3 DAP1 staining of chloroplasts, protoplasts and amyloplasts

Chloroplasts were prepared by differential and density gradient centrifugation as detailed in section 4.2.2. Amyloplasts were prepared from protoplasts provided by Sheila Baird (I.C.I., Runcorn). Both were stained with the fluorescent dye, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), to locate nDNA contamination of chloroplast preparations and to observe plastid nucleoids (Kapuścińsky and Szer, 1979; Selldén and Leech, 1981).

Chloroplast DAPI staining

Samples were taken from various bands of sucrose gradients (Fig. 4.3.4.1) and were DAPI stained on ice, in the dark, for 10-15 min, in the ratio 1:5 v/v sucrose gradient: DAPI (Sigma) at 5 μg/ml in Medium B (section 4.2.2). The chloroplasts were pelleted at 2,300 x g and resuspended in Medium B containing 3.5% glutaraldehyde (BDH).

Protoplast preparation

Grains were harvested from greenhouse grown plants at 10-13 d.p.a. Nine endosperms were excised and incubated in 3

ml of filter-sterilised Incubation buffer: 10 mM 2-(N-morpholino)ethane sulfonic acid (MES, Sigma), 0.2M sucrose, 0.3M mannitol, 0.1% w/v bovine serum albumin (BSA), 1 mM CaCl, 10 mM $MgCl_2$ and 60 mM KCl, pH 5.5, containing 1% w/v pectinase (Serva) and 2% w/v cellulase; either at 4°C overnight or at 25°C for 2 h. Incubation buffer was replaced with 0.5 ml of modified Incubation buffer containing 0.5 M mannitol and omitting the enzymes and sucrose. The softened tissue was passed through a Pasteur pipette tip three times, filtered through a 240 μm mesh attached to a syringe and centrifuged on a 90% Percoll (a polyvinylpyrolidone-coated colloidal silica suspension, Sigma) gradient containing 0.5M mannitol.

Amyloplast DAPI staining

Protoplasts were left on ice for 15-30 min to settle under gravity; the supernatant was discarded and the sediment resuspended by swirling in Medium M: 10 mM MES, 500 mM mannitol, 60 mM KCl, 0.1% BSA, pH 5.5. Protoplasts were DAPI stained at 4°C, in the dark, using three different procedures: (i) 1 ml of protoplasts were stained directly with 25 μl of 1 mg/ml DAPI, (ii) 0.5 ml of protoplasts were incubated in 0.5 ml of 2.2 mg/ml digitonin, for 5 min and then DAPI added, as for (i), finally, (iii) 0.5 ml of protoplasts were incubated in digitonin, as for (ii), 2.5 μl of 10 mg/ml DNase-1 was added, the mixture incubated for 10 min and then DAPI stained, as for (i). Each treatment was incubated with DAPI for 20 min, the supernatant discarded and

the sedimented material resuspended in Medium M containing 5% glutaraldehyde. The sediments were once more left to settle and were resuspended as before.

Fluorescence microscopy

Protoplasts and organelles were viewed using either a Leitz Orthoplan or an Orthoplan II microscope, with fluorescent illumination. Excitation filter UG1 provided light from 300-400 nm and suppression filters K400 or 430 reduced background. Photographs were taken using a Leitz Orthomat camera, initially using Kodak TriX Pan (chloroplasts) or Kodak CM (amyloplasts) films which were push processed to 1,600 ASA (Selldén and Leech, 1981). However, finer results were obtained by not push processing, using Ilford HP5 (amyloplasts and chloroplasts).

4.2.4 Preparation, extraction and purification of cloned ctDNA

Plasmid constructs of pBR322 containing ctDNA SalI, BamHI and PstI restriction fragments, which together comprised the whole plastome, were generously provided by Cathy Bowman (at P.B.I., Cambridge). The plasmid pTA71 (Gerlach and Bedbrook, 1979), provided by Wolf Schuch (at I.C.I., plc, Runcorn), comprised wheat DNA enriched for ribosomal genes cloned into pAC184.

Transformation of E. coli

L-broth (Luria broth): 1% w/v Bacto-tryptone (Difco),

0.5% w/v Bacto-yeast extract (Difco) and 0.5% w/v NaCl, pH 7.2 with HCl; was inoculated with Escherichia coli strain C600 or HB101 (Boyer and Rouilland-Dussoix, 1969) and incubated at 37°C overnight. The stock was subcultured 1:100 v/v in L-broth and shaken at 37°C until culture absorbance at 500 nm had reached 0.5±0.1 (approx. 100 min for C600 and $3\frac{1}{2}$ h for HB101), at which point the bacteria were estimated as being mid log phase. They were pelleted by centrifugation at 2500 x g, 4°C, for 5 min in a MSE Chilspin and the supernatant was replaced by 0.5 vol of 0.1 M $CaCl_2$ at 4°C. The mixture was shaken gently, incubated on ice for 10 min, centrifuged as before and the supernatant replaced by 0.2 vol of 0.1 M $CaCl_2$. The bacteria were resuspended as before and incubated on ice for at least 1 h, by which time they were considered to be competent to take up extraneous DNA (Dagert and Ehrlich, 1979).

Plasmid DNA was added in 2, 20 and 200 ng amounts to 200 µl aliquots of the competent cells, to ensure transformation at a suitable frequency (Bedbrook, 1982). The mixtures were incubated on ice for 45 min, then heat shocked at 42°C, for 2-3 min, and returned to ice for 0.5-1 h; after which 800 µl of L-broth was added to each and the bacteria incubated at 37°C for 1 h, without shaking. The cells were plated onto L-agar: 1.5% w/v agar in L-broth and 0.1% w/v D-glucose; containing an appropriate antibiotic. Bacteria transformed by plasmids containing PstI restriction fragment inserts grew on 10 µg/ml tetracyclin (HCl, Calbiochem-Behring Corp., U.S.A.) and those transformed by plasmids containing either

BamHI or SalI inserts grew on 100 μg/ml ampicillin (Na-salt). Bacteria were also plated onto L-agar containing the opposite antibiotic to ensure there were no recombinant plasmids. Agar plates were incubated inverted at 37°C, overnight. For ctDNA cloning see Rawson and Andrews (1982).

Batch culture of transformants

Three colonies for each plasmid were picked, separately, into 10 ml of L-broth containing either 10 μg/ml tetracyclin (PstI inserts) or 100 μg/ml ampicillin (BamHI or SalI inserts). These were incubated with shaking, at 37°C, for 8 h after which 3 ml was subcultured into 300 ml of antibiotic containing L-broth and incubated as before, overnight. Bacteria were also withdrawn and diluted 1:1 v/v in 80% sterile glycerol (or 1:2 v/v in 60%) (AnalaR, BDH) and stored at -20°C as stocks for later use.

Alkaline Extraction of plasmid DNA

Plasmid DNA was extracted using a modification of the procedures described by Maniatis et al. (1982). Bacteria were harvested from overnight cultures by centrifugation in a MSE18 at 10000 x g, 4°C for 5 min. The supernatant was replaced by 0.1 vol of Solution I: 25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA and 2 mg/ml lysozyme (Grade I), pH 8.0; the pellet was thoroughly resuspended and the mixture incubated at 4°C for 5 min. Then 2 vol of Solution II: 0.2 M NaOH and 1% SDS; was added and the solutions were gently mixed and incubated, as before. After 5 min 0.5 vol of

Solution III: 5 M potassium acetate, pH 4.8; was added and the mixture again placed on ice for 5 min. (Solution III was prepared by adding glacial acetic acid to 60 ml of 5 M potassium acetate, until the pH reached 4.8, and the volume was made to 100 ml with distilled water.)

Bacterial cell debris, including chromosomal DNA, was pelleted by centrifugation at 4°C, 8000 x g, for 10 min, the supernatant decanted and nucleic acid was ethanol precipitated at -80°C, as described in section 3.2.1. The precipitate was pelleted by centrifugation at 4°C, 10000 x g, for 10 min, vacuum dried for 10 min and resuspended in TE.

Phenol extraction of plasmid DNA

Protein contamination was reduced by phenol and chloroform extractions. The plasmid preparation was diluted with 0.1 vol of 3 M sodium acetate, phenol extracted twice using ultrapure phenol (Redistilled Nucleic Acid Grade, BRL, Bethesda Research Laboratories, U.K., Ltd.) buffered to pH 8.0 using 10 x TE, containing 0.1% w/v 8-hydroxyquinoline, and extracted twice with chloroform. Each extraction was centrifuged at 13000 x g for 5 min and the upper aqueous layer retained (see section 3.2.1). Nucleic acid was ethanol precipitated, as described in section 3.2.1, and resuspended in distilled water.

Restriction enzyme digestion and RNase treatment

Plasmids were restricted with the appropriate restriction enzymes to liberate the ctDNA inserts. All

enzymes came from Amersham Intl., plc. RNA contamination was reduced by ribonuclease (RNase) treatment followed by phenol: chloroform extraction.

Plasmids containing PstI inserts were digested in Medium salt buffer: 30 mM Tris-HCl, 5 mM NaCl, 7.5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT), pH 7.5; to which PstI was added at $\geq$8 units/μg DNA. Plasmids containing BamHI or SalI inserts were digested in High salt buffer: 30 mM Tris-HCl, 10 mM NaCl, 7.5 mM $MgCl_2$ and 1 mM DTT, pH 7.5; to which BamHI was added at $\geq$10 units/μg DNA or SalI at $\geq$6 units/μg DNA. The mixtures were incubated at 37°C for 2 h then 0.15 mg/ml RNase A (Type III) was added and the digestions continued for 1 h. RNase A was prepared at 10 mg/ml and heated, before use, to 100°C for 15 min, to destroy DNase activity.

Plasmids were then phenol extracted; phenol: chloroform 1:1 v/v extracted, to remove RNase (Maniatis et al., 1982); chloroform extracted and ether extracted. Each extraction was centrifuged at 13000 x g for 5 min and the aqueous layer retained. Plasmid fragments were ethanol precipitated (as described in section 4.2.1) and resuspended in TE.

Electrophoresis and electroelution

Plasmids used for Southern blots (section 4.2.5) were not purified further since pBR322 and any residual RNA would be separated from the ctDNA fragments during electrophoresis. Plasmids containing fragments P6 and B2, which were nick translated and hybridised to Southern and dot blots (sections 4.2.7, 4.2.8, and 4.2.9), were prepared as described above,

electrophoresed (Hallick et al., 1982) and the ctDNA fragments isolated by electroelution (Maniatis et al., 1982). However, for a few dot blots the intact plasmid was nick translated.

Loading buffer: 25% v/v glycerol, 5 x TBE, 0.1% w/v bromophenol blue (Fisons) and 0.1% w/v xylene cyanal F.F., pH 8.3; was added 4:1 w/v to the restricted plasmids which were electrophoresed through an agarose slab gel: 1% w/v agarose (Type II Med EEO), 1 x TBE (TBE is 0.1 M Tris-HCl, 0.10 M boric acid, 2 mM EDTA, pH 8.3) and 0.6 μg/ml ethidium bromide. The gel was prepared in an horizontal submerged gel apparatus (series 1025, Model H4, BRL) and run in TBE at 200 V for 3 h. The bands were visualised under u.v. light and those containing ctDNA fragments were excised. Dialysis tubing was boiled in 1 mM EDTA for 20 min and rinsed in TBE. Each gel slice was clipped inside a short length of the tubing, containing TBE, and air bubbles were squeezed out (using medi-clips', Sartorius). The dialysis bag was immersed in an 0.5 cm layer of TBE in a minigel apparatus (Cambridge Biotechnology Laboratories) and DNA was electrophoresed out of the gel at 100 V for 1.5 h. The current polarity was reversed for 30 s, to draw DNA off the dialysis tubing and the TBE was withdrawn. DNA was recovered by ethanol precipitation (section 4.2.1).

4.2.5 Extraction of nuclear DNA

Nuclear DNA was prepared from wheat embryo nuclei which were isolated as described by Luthe and Quatrano (1980).

Preparation of wheat nuclei

Wheat embryos were dissected out of growing wheat grains from spikes aged from 20 to 50 d.p.a. (cv Timmo) (section 2.2.4). Nuclei were extracted at 4°C using glassware heated in 1% w/v sodium lauryl sulphate (SDS) at 60°C for 15 min and rinsed in sterile distilled water.

Embryos (2 g) were homogenised 1:2 w/v in a modified Honda buffer: 25 mM Tris-HCl, 0.44 M sucrose, 10 mM $MgCl_2$, 2.5% w/v Ficoll (mol. wt. 400,000), 5.0% Dextran T70 (Pharmacia Fine Chemicals), 2 mM spermine (N,N'-bis[3-aminopropyl]-1,4-butandiamine), 10 mM 2-mercaptoethanol, and 0.5% w/v Triton X-100, pH 7.6 (Honda et al., 1966); by grinding with a chilled mortar and pestle for 1 min. The homogenate was diluted with 2 vol of Honda buffer and filtered through four layers of cheesecloth and two nylon meshes (80 and 61 μm, Henry Simon Ltd.). The retained solid was reground and filtered as before and the filtrate was centrifuged at 4°C, 5800 x g, for 5 min.

The pelleted nuclei were gently resuspended in Honda buffer without spermine and the suspension was centrifuged in a discontinuous gradient of Percoll. Percoll gradients were prepared by sequentially layering 7.5 ml of 80, 60 and 40% v/v Percoll, in 25 mM Tris-HCl, 0.44 M sucrose and 10 mM $MgCl_2$, pH 7.5, onto a 2 M sucrose cushion in the same solution. The gradients were centrifuged in a Beckman SW28 swing out rotor at 4°C, 4100 x g for 30 min. Most nuclei banded just above the sucrose cushion. The band was

withdrawn and diluted with 2 vol of Honda buffer without spermine and centrifuged at 4°C, 4800 x g for 5 min. They were resuspended and centrifuged once more to remove Percoll.

Photography of nuclei

Nuclei taken from Percoll gradients were pelleted and resuspended as described and stained with ethidium bromide or DAPI to determine whether they were disrupted or contaminated (see section 4.2.3). In addition, the samples were stained with iodine to visualise any contaminating starch. The tissue was fixed in 5% v/v glutaraldehyde in Honda buffer and examined using light and fluorescence microscopy. Photographs were taken using an Olympus OM-2 attached to the microscope, and Ilford XPI film.

Extraction of nuclear DNA

Nuclear DNA was extracted from isolated nuclei as described by Bedbrook et al. (1980). The pelleted nuclei were resuspended in 5 ml of Buffer T (described in section 4.2.1) and incubated at 37°C for 2 h. DNA was separated from RNA and protein by CsCl density gradient centrifugation, followed by butanol extraction and ethanol precipitation (section 4.2.1). DNA banded in a single band on CsCl gradients.

HpaII digestion of nuclear DNA and electrophoresis

Because dot blot results suggested that there was plastid DNA (ptDNA) contamination of the nDNA, the DNA was

purified further by digestion using the methylation sensitive restriction enzyme HpaII (Kessler and Höltke, 1986; Gruenbaum et al., 1981a,b), to selectively digest any ptDNA (Timmis and Steele Scott, 1983). NDNA was digested in Low salt buffer: 30 mM Tris-HCl, 7.5 mM $MgCl_2$ and 1 mM DTT, pH 7.5; to which HpaII (BRL) was added at 7 units/μg DNA. The mixture was incubated at 37°C overnight. An 0.2 vol of loading buffer: 5% w/v SDS, 25% v/v glycerol and 0.025% w/v bromophenol blue; was added to the sample which was electrophoresed through an agarose slab gel: 0.5% w/v agarose, TBE and 0.6 μg/ml ethidium bromide. The gel was run in TBE at 30 V, overnight (in a Bromma 2012 Maxiphor, LKB, apparatus). A HindIII digest of lambda DNA was run alongside as a molecular weight marker. This was prepared by digesting 2.5 μg of lambda DNA (5250SA, BRL) in Core Buffer (BRL) to which HindIII (Amersham) was added at 18 units/μg DNA, at 37°C for 1 h.

Photography of agarose gels

DNA was visualised over u.v. light from a transilluminator and photographs were taken using a Polaroid MP-3 land camera equipped with a 75 mm lens and either coaterless type 667 or positive/negative, type 665, land pack film (Polaroid). Using film type 667 optimum results were obtained when the gel was well destained, in TBE, and photographed using yellow (no.8) and red gelatin lens filters (Wratten, Kodak) with an f stop of 5.6 and exposure time of 20-25 s. (Yellow filter alone: f11 and 3 s exposure). Film type 665 was exposed for 15-20 s at f4.5 (Hallick et al.,

1982). Film was developed for 30-60 s.

Electroelution onto dialysis membrane

Rather than electroelute in a dialysis bag (section 4.2.4), high molecular weight nDNA was recovered by electroelution directly onto a strip of dialysis membrane (Yang et al., 1979). The tubing was prepared as described in section 4.2.4, cut open and inserted into a slit in the gel below the high molecular weight band of DNA. Electrophoresis was resumed at 100 V until all DNA of more than 16-20 kb had contacted the dialysis membrane, as could be seen under u.v. light. The DNA was rinsed off the membrane, using TBE, and filtered through a siliconised glass wool column to remove any contaminating agarose. The DNA was recovered by ethanol precipitation (section 4.2.1).

4.2.6 Quantitation of DNA and RNA

For quantitative experiments such as dot blots and reassociations, RNA and DNA concentrations were estimated from the absorbance of nucleic acid at 260 and 280 nm (Maniatis et al., 1982). In addition, DNA concentration was verified by either diaminobenzoic acid (DABA: 3,5-diaminobenzoic acid hydrochloride, Aldrich Chem. Co., Inc.) or diphenylamine assay (Vytasek, 1982; Giles and Myers, 1965), since a number of chemicals commonly used for nucleic acid extractions also absorb below 300 nm, as do starch and protein. When DNA concentration was less critical, such as for Southern blots, electrophoresis in an agarose minigel not

only indicated DNA concentration but also integrity and purity (Maniatis et al., 1982).

Spectrophotometric determination of DNA or RNA

Measured amounts of a sample were diluted in distilled water to <0.07 mg/ml DNA or RNA and the absorbance spectrum from 300-200 nm was recorded using a Pye Unicam SP8-100 ultraviolet spectrophotometer. DNA concentration was estimated by extrapolation from a nomograph (Warburg and Christian, 1942).

Pure preparations of DNA and RNA have A_{260}:A_{280} ratios of 1.8 and 2.0, respectively. For pure samples an absorbance of 1.0 corresponds to approximately 50 μg/ml double stranded DNA or 40 μg/ml single stranded DNA or RNA (20 μg/ml oligonucleotides) (Maniatis et al., 1982).

DABA assay procedure

Measured volumes of a DNA sample were diluted to 50 μl in 1 M ammonium hydroxide (or NaOH) and oven dried at 60°C for 3 h, after which 100 μl of 1 M HCl was added and the tube vortexed. An 1.32 M DABA solution in distilled water was prepared immediately before use and 100 μl added to each sample. The mixture was incubated in a waterbath at 60°C for 30 min, cooled for 5 min and 1 ml of 1 M HCl was added.

A standard curve was prepared from seven duplicated solutions of calf thymus DNA (type I), containing from 0.1-25.0 μg/ml DNA. Fluorescence was measured using an Aminco-Bowman spectrophotofluorometer, with excitation and

emission wavelengths set at 405 and 520 nm, respectively.

Diphenylamine assay procedure

The procedure used is described fully in section 2.2.5.

Agarose minigel electrophoresis

Agarose gels containing 0.7-1.0% agarose in TBE were prepared using a minigel apparatus. Nucleic acids were stained by either including 0.5 μg/ml ethidium bromide in the gel or by immersing it, after electrophoresis, in 0.5 μg/ml ethidium bromide in TBE, for 30 min, and destaining in TBE for 30 min. Samples were loaded in 0.2 vol of Loading buffer (sections 4.2.4 and 4.2.5) and were electrophoresed through the gel at 70 V for 1-2 h, after which the gel was photographed, as described in section 4.2.5. To quantify DNA an HindIII digest of lambda DNA of known concentration, prepared as described in section 4.2.4, was electrophoresed alongside the samples. The amount of DNA in each fragment band could be related to intensity of fluorescence (Maniatis et al., 1982).

4.2.7 Nick translation

DNA was radiolabelled by nick translation using a BRL Nick Translation Reagent Kit (Rigby et al., 1977). CtDNA used for reassociations (section 4.2.8) was nick translated using [^{3}H]TTP ([methyl-^{3}H] thymidine 5'-triphosphate, ammonium salt, 43 Ci/mmole, Amersham). CtDNA fragments P6 and B2, hybridised to Southern blots (section 4.2.8) and slot

blots (section 4.2.10), were nick translated using [^{32}P]dATP (deoxyadenosine 5'-[γ-^{32}P] triphosphate, triethylammonium salt, aqueous solution, 10 mCi/ml, Amersham). CtDNA fragments P6 and B2 (Fig. 4.3.2.1), hybridised to DNA dot blots (section 4.2.9), were nick translated using [^{35}S]dCTPαS (deoxycytidine 5'-(α-[^{35}S]thio)-triphosphate, triethylammonium salt, 650 Ci/mmole, Amersham), as were nDNA and total endosperm DNA, hybridised to Southern blots of restricted plasmids (section 5.2.5).

Nick translation using [^{3}H]TTP

15 μCi of [^{3}H]TTP was dried under nitrogen and dissolved in 1.8 μl of 1 mM dCTP (2'-deoxycytidine 5'-triphosphate, Na salt), 1.8 μl of 1 mM dGTP (2'-deoxyguanosine 5'-triphosphate Na salt), 1.8 μl of 1 mM dATP (2'-deoxyadenosine-5'-triphosphate, Na_2 salt), 1.5 μl of 1 mM TTP (thymidine 5'-triphosphate) and 10 μl of 10 x Reaction buffer: 0.5 M Tris-HCl, 50 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol and 0.5 mg/ml BSA (nuclease free from BRL), pH 7.8. CtDNA was ethanol precipitated, as described in section 4.2.1, and resuspended in distilled water, because EDTA inhibits the nick translation reaction. Up to 2 μg of ctDNA was added to the nucleotides, the volume made up to 96 μl with distilled water and the mixture incubated at 15°C. The reaction was started by adding 2 μl of 100 ng/ml DNase 1 for 10 min, followed by 2 units of DNA polymerase 1 (from E. coli) and the incubation continued for 1 h. The reaction was terminated by adding 10 μl of 0.3 M EDTA.

Nick translation using [^{32}P]dATP

To obtain DNA of at least 10^8 c.p.m./μg only 80-120 ng of DNA was nick translated. On ice, DNA was mixed with 5 μl of Solution A_1: 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 0.5 M Tris-HCl, 50 mM $MgCl_2$, 0.1 M 2-mercaptoethanol and 100 μg/ml BSA, pH 7.8; 40 μCi of [^{32}P]dATP and 5 μl of Solution C: 0.4 units/μl DNA polymerase 1, 40 pg/μl DNase 1, 50 mM Tris-HCl, 5 mM magnesium acetate, 1 mM 2-mercaptoethanol, 0.1 mM PMSF (phenylmethylsulphonylfluoride), 50% glycerol and 100 μg/ml BSA, pH 7.5. The mixture was made up to 50 μl with distilled water and incubated at 15°C for 60 min. The reaction was terminated as before.

Nick translation using [^{35}S]dCTPαS

DNA was nick translated as above, except that Solution A_1 was replaced by A_2 containing dATP, dGTP and TTP. In addition, 7.5 μl of Solutions A_2 and C were added, rather than 5 μl, to improve nick translation efficiency, and the mixture was incubated at 15°C for 2 h.

Sephadex G50 chromatography

Carrier DNA (calf thymus or salmon testes) was added to the terminated nick translation mixture to give a DNA concentration of 1 μg/50 μl. Sephadex G50 fine (Pharmacia Fine Chemicals) was equilibrated in 10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0) and packed in either a 13 x 0.8 cm column or a pasteur pipette plugged with silicon coated glass wool. DNA and nucleotides were eluted from the column using the same

buffer and 0.6 ml fractions were collected in microfuge tubes. A fraction collector (Bromma 212 Redirac, LKB) was used with the larger column. For ^{35}S and ^{3}H nick translations, 2 μl subsamples were withdrawn, diluted in 2 ml of 0.5% w/v PPO (2,5 diphenyloxazole, BDH) in triton X100: toluene 3:7 v/v in 20 ml polyethylene vials and counted in a LKB Wallac 1217 Rackbeta liquid scintillation counter. The ^{32}P nick translated fractions could be monitored using a hand held γ counter (Series 900 Mini-Monitor, Mini-Instruments, Ltd.) and DNA and nucleotide peak fractions were determined: the former were pooled and stored at -20°C. The presence of radiolabelled DNA was verified, when necessary, by precipitation in TCA as described in section 4.2.9. For ^{3}H and ^{35}S nick translations, dextran blue (av mol wt. 2,000,000) and methylene blue were sometimes passed through the Sephadex G50 column to show where the peak fractions of DNA and nucleotides, respectively, were likely to be eluted.

4.2.8 Southern blots of total DNA

PstI digestion and agarose gel electrophoresis

Chloroplast, total leaf and total endosperm DNA samples were digested with the methylation sensitive enzyme PstI (Gruenbaum et al., 1981b), which restricts ptDNA leaving nDNA largely unrestricted (Bowman and Dyer, 1982). PstI was added at 15 units/μg of DNA in Core buffer (section 4.2.5) and incubated at 37°C for 2 h. Digests were diluted 4:1 v/v with Loading buffer (section 4.2.5) and electrophoresed through an

0.8% w/v agarose gel (section 4.2.4), at 200 V for 3 h. Gels were photographed as described in section 4.2.4.

Southern blot procedure

Gels were immersed in 1.5 M NaCl, 0.5 M NaOH; at room temperature for 45 min, and then transferred to 0.5 M Tris-HCl and 1.5 M NaCl, pH 7.5; for 45 min. A glass plate was supported over a developing tray containing 1.5 l of 20 x SSC (section 4.2.1) and three sheets of Whatman 3 mm filter paper were laid across the plates, with both ends immersed in the solution (Southern, 1975; Bingham and Hallick, 1982). A gel was placed onto the filter paper and plastic strips laid around the edges, to prevent contact between the lower and upper layers of filter paper. A nitrocellulose filter (BA85, 0.8 μm, Schleicher and Schuell, GmbH) was cut to the same dimensions as the gel and was placed on top, avoiding air bubbles. The filter was overlaid with six sheets of Whatman 3MM cut to the same size, the first soaked in 20 x SSC. A 10 cm stack of paper hand towels was placed on top and weighted down. The gel was left to blot for 16 h, then dismantled and the filter air dried on Whatman 3MM before being baked at 80°C for 2 h in a vacuum oven.

Hybridisation

Filters were first prehybridised to reduce non-specific binding of radiolabel, and then hybridised to the radiolabelled probe (Fluhr et al., 1982). Hybridisation buffer was prepared containing: 5 x SSPE (1 x SSPE is 0.18 M

NaCl, 10 mM sodium phosphate buffer, pH 8.3 (Gomori, 1955) and 1 mM EDTA), 0.1 mg/ml herring sperm DNA, 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% w/v BSA (Fraction V, Boehringer Mannheim), 0.02% w/v Ficoll (mol wt. 400000) and 0.02% w/v PVP (mol wt. 40000), Denhardt (1966)) and 0.01% w/v SDS. Herring sperm DNA was prepared at 10 mg/ml, sonicated for 15 min, as described in secton 4.2.9, and denatured at 100°C for 10 min, before use. Each filter was sealed into a plastic bag, using a heat sealer (Lakeland Plastics Ltd.) with 10 ml of Hybridisation buffer/100 cm^2 of filter. The bag was wrapped in damp paper towels, sealed into a second bag and incubated in an air circulating oven (Gallenkamp Orbital Incubator, illuminated, cooled) at 65°C for 4-6 h. Hybridisation buffer was then poured away and replaced with the same buffer containing either 20 ng of [^{32}P]ctDNA fragment P6 or 100 ng of [^{32}P]ctDNA fragment P7 (section 4.2.7) at 5 ml of buffer/100 cm^2 of filter. Radiolabelled DNA was denatured immediately before use, at 100°C for 10 min. Fluid was spread evenly over the filter which was incubated as before for a further 16-20 h.

Filters were washed in 3 x 0.5 l of 3 x SSC, at 65°C, for 3 x 1 h, and then air dried on Whatman 3MM filter paper.

Autoradiography

X-ray film (Fuji NIF RX100) was exposed to each filter in an autoradiograph cassette (Genetic Research Instrumentation Ltd.) with an intensifying screen (Lighting plus, extra life, E.I. Du Pont de Nemours and Co., Inc.)

placed on the opposite side of the filter to the film (Laskey, 1980). Cassettes were left at -80°C, overnight or for several days. Film was developed in Kodak D-19 developer for 30 s-5 min, depending on film exposure, rinsed in 1% acetic acid 'stop' for 2 min, and fixed in Kodak Unifix for 4 min, at room temperature.

4.2.9 Reassociation of plastid DNA

Sample preparation

Total leaf and endosperm DNA were extracted as described in section 4.2.1 and ctDNA as described in section 4.2.2. DNA concentration was assayed by absorbance scans and DABA assay (section 4.2.6). Total endosperm and leaf DNA were diluted to 110 μg/0.5 ml Buffer R: 0.03 M Tris-HCl, 1.0 M $NaClO_4$, 0.1 mM EDTA and 0.1% w/v Sarcosyl, pH 8.) (Lamppa and Bendich, 1979a); and sonicated on ice at 37 watts (maximum noise), for 2.5 min, using a sonicator (Ultrasonics Ltd.) with a 4.5 mm probe. Samples were cooled for 15 s after each 15 s sonication. CtDNA was sonicated and then nick translated using [^{3}H]TTP, as described in section 4.2.7. DNA was sonicated to a uniform size to enable comparison of reassociation rate between samples.

Reassociation procedure

DNA samples were reassociated as described by Lamppa and Bendich (1979a). [^{3}H]ctDNA was added to total DNA samples in microfuge tubes to final concentrations of 0.04 μg/500 μl [^{3}H]ctDNA and 100 μg/500 μl total DNA in Buffer R. Standards

containing 5 μg/500 μl ctDNA and 0.04 μg/50 μl [^{3}H]ctDNA were also prepared. Two 20 μl subsamples were withdrawn and the mixture was overlaid with sterile paraffin oil. The microfuge tubes were incubated at 100°C for 10 min, to denature the DNA and 20 μl subsamples were withdrawn at 7.5 and 9 min. The microfuge tubes were transferred to a waterbath at 60°C and 20 μl subsamples withdrawn at log intervals from 15 s. Subsamples were immediately placed in an ice bucket containing solid CO_2 and ethanol, to freeze rapidly, and were stored at -20°C.

S_1 nuclease digestion

The S_1 nuclease digestion procedure was adapted from that described by Maxwell et al. (1978). Preliminary experiments were carried out to determine the optimum conditions for S_1 nuclease digestion and are discussed in Appendix II.

Measured volumes, 20 μl at room temperature, were withdrawn from the stored subsamples, diluted with 180 μl of distilled water and 210 μl of 2 x S_1 nuclease buffer (1 x S_1 nuclease buffer is 25 mM sodium acetate, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 5 mM 2-mercaptoethanol, pH 4.4), and 10 μl of S_1 nuclease (from Aspergillus oryzae type III) at 0.5 units/μl in S_1 nuclease buffer, was added. Carrier DNA (calf thymus at 0.5 mg/ml) was added to subsamples from reassociations containing less than 100 μg/500 μl DNA, to bring the DNA concentration to 4 μg/20 μl S_1 nuclease buffer. Samples were incubated at 37°C for 2 h and then stored

frozen. Double stranded DNA was assayed either by spotting samples onto DEAE-cellulose filters (DE-81 filters, 2 cm diameter, Whatman) (Maxwell et al., 1978) or by TCA (SLR, Fisons) precipitation (Maniatis et al., 1982). Experiments carried out to determine the optimum conditions for both assay procedures are discussed in Appendix III.

Assay of double stranded DNA using DE-81 filters

DE-81 filters were wetted in 0.48 M sodium phosphate buffer, pH 6.8 (Gomori, 1955), semi-dried and spotted with half a S_1 nuclease incubated sample, semi-dried and spotted with the remaining volume of sample. Filters wre dried, rinsed for 3 x 1 min in three washes of 20 ml per filter, then dried completely in an oven at 40°C. Filters were placed sample side uppermost in 20 ml scintillation vials, 3 ml of 0.05% w/v PPO in toluene was added and the sample counted in a Packard Tri-Carb liquid scintillation spectrometer.

Assay of double-stranded DNA by TCA precipitation

S_1 nuclease digested samples were added 1:10 v/v to 20% TCA and left at 4°C overnight. Precipitated DNA was filtered onto glass fibre discs (GFC or GFB, 2 cm diameter, Whatman) using a Millipore sampling manifold filter holder (Millipore Corporation, U.S.A.). The filters were positioned smooth surface uppermost and prewetted with ice cold 10% TCA before filtering the samples slowly under vacuum: they were each rinsed with 30 ml of ice cold 5% TCA, 10 ml of ice cold 95% ethanol and 3 ml of ether. Filters were left to air dry

before being counted as before.

4.9.10 Dot and slot blots

Dot and slot blots were carried out using a modification of the original procedure described by Kafatos et al. (1978).

Sample preparation

Each filter was prepared bearing two rows of four or five individual dilutions for each total endosperm DNA sample. In order to assign absolute values for ptDNA content of samples, internal standards were included on each filter: these usually consisted of one or two rows of probe DNA, each containing eight individual dilutions, as well as two rows of four to eight individual dilutions of total leaf DNA. Duplicate or triplicate filters were prepared using the same dilutions: these were probed with ctDNA fragments P6 or B2, pTA71 or ctDNA.

When intact pTac plasmids, containing either P6 or B2, were used as standards, rather than the excised fragments, the plasmids were fragmented by sonication. Each was sonicated in 0.5 ml of distilled water, as described in section 4.2.9, before being denatured. CtDNA was heated to 65°C for 10 min in 0.2 M NaOH in order to render any supercoiled molecules either open circular or linear. PtDNA in total endosperm and leaf DNA samples was judged to be linear already since only one band was visible on CsCl gradients (Fig. 4.1). The ptDNA was possibly linearised during protease digestion, due to endogenous DNase, or due to

vigorous phenol and chloroform extractions (section 4.2.1).

Sample concentrations were estimated from their absorbance spectra and by diphenylamine assay (section 4.2.6). Subsamples were diluted in sterile distilled water to appropriate concentrations, an equal vol of 4 M NaOH was added, and the samples left for 20 min to denature fully. An equal vol of 2 M acetic acid was added, to bring the pH to 7.0, and the samples were placed on ice. Subsamples of decreasing volume were withdrawn, diluted to 30 or 50 μl in distilled water, in a microtitre plate (Nunclon, Nunc, Denmark), and diluted finally 1:3 v/v with 20 x SSC. Each sample was mixed thoroughly.

Filters used were either nitrocellulose (BA85, 0.85 μm, Schleicher and Schuell, West Germany) or Biodyne A (P/N BNRG 137, 0.2 μm, or P/N BNNG 2225, 1.2 μm, Pall Europe, Ltd.), in either a slot blot (Minifold II, Schleicher and Schuell) or a dot blot apparatus (Minifold SRC-96-D filtration manifold) (White and Bancroft, 1982). Filters were handled wearing gloves; they were prewetted in distilled water for 2 min, then soaked in 15 x SSC for 45 min, before use. They were supported on one or two blotting papers (GB003, Schleicher and Schuell) and clamped inside the apparatus which was attached to a water vacuum so that samples were drawn through at a steady rate.

Samples measuring either 50 or 100 μl were pipetted from the microtitre plate into each well and rinsed through with 100 or 200 μl of 15 x SSC (volumes varied between but not within filters). Filters were placed on Whatman 3MM filter

paper to air dry and then baked at 80°C under vacuum: nitrocellulose for 2 h and Biodyne A for 1 h.

Hybridisation

Probe DNA was nick-translated as described in section 4.2.7. All slot blots, probed with [^{32}P]DNA, and the earlier dot blots, probed with [^{35}S]DNA, were prehybridised and hybridised as described in section 4.2.8, except that only 4 ml of Hybridisation buffer was used for each 100 cm^2 of membrane. For later dot blots, probed with [^{35}S]DNA, the Hybridisation buffer was modified to contain 0.5 mg/ml salmon testes DNA, instead of the previous 0.1 mg/ml. Filters were prehybridised and hybridised for periods of 3-14 and 16-40 h, respectively. Hybridisation solution was mixed around the filter several times during the incubation.

For filters probed with [^{32}P]DNA three washes in 3 x SSC, as described in section 4.2.8, removed any trace of non-specific binding of ^{32}P from the autoradiographs. However, using [^{35}S]-labelled probes, which gave high backgrounds at this stringency, better results were achieved using a more stringent and vigorous procedure (Biodyne A filters only). Filters were rinsed in 2 x SSC and 0.1% w/v SDS; resealed into a bag containing 400 ml of the same solution per 100 cm^2 of membrane and shaken at 200 rpm at room temperature for 5 min. The wash was repeated a further three times, then the filters were incubated in 0.1 x SSC and 0.1% w/v SDS; at 50°C for 2 x 15 min, using 2 x 400 ml/100 cm^2 of membrane. Filters were either air dried on Whatman

3MM, or wrapped immediately in cling film: wet filters could be rewashed, if necessary, or reprobed (Cannon et al., 1985).

Autoradiography and preflashing

Filters were taped onto Whatman 3MM filter paper, spotted with radioactive ink and autoradiographed using Kodak X-Omat film, as described in section 4.2.8: intensifying screens were not used for [^{35}S]-probed filters (Laskey, 1980). A number of films were preflashed before use in order to bypass the reversible first stage of latent image formation in the film (Laskey and Mills, 1975). A curve was prepared of autoradiograph absorbance at 540 nm versus distance of the flash gun (Solarglow) from the film, which was attached to the wall. A distance of 1.30 m gave an absorbance of 0.15 above background.

Densitometric measurement of autoradiographs

Autoradiographs of slot blots were scanned using a MKIIIC Double Beam Recording Microdensitometer (Joyce Loebel and Co., Ltd.) using wedge D159, spot width 115 and slit width 40.7. Absorbance was measured along a line perpendicular to the slots. Peak height was taken as the measure of hybridisation. Autoradiographs of dot blots were scanned using a Joyce Loebel Chromoscan 3 using transmission mode, a 626 nm filter and a slit width of 0.3 cm. Peak area was taken as the measure of hybridisation (Crossway and Houck, 1985).

Liquid scintillation and Čerenkov counting

Filters were dried completely before being dissected and autoradiographs were used to locate spot positions. Slot blots were divided into rectangles of equal size. A perspex plate with parallel slots was made to facilitate cutting dot blots into squares, using a scalpel. Filter squares were placed in 20 ml scintillation vials with the spotted surface uppermost. Filters probed with [^{32}P]DNA were counted initially without scintillant (Kellogg, 1983). All filters were counted in 0.5% w/v PPO in toluene, as described in section 4.2.7.

4.3 Results

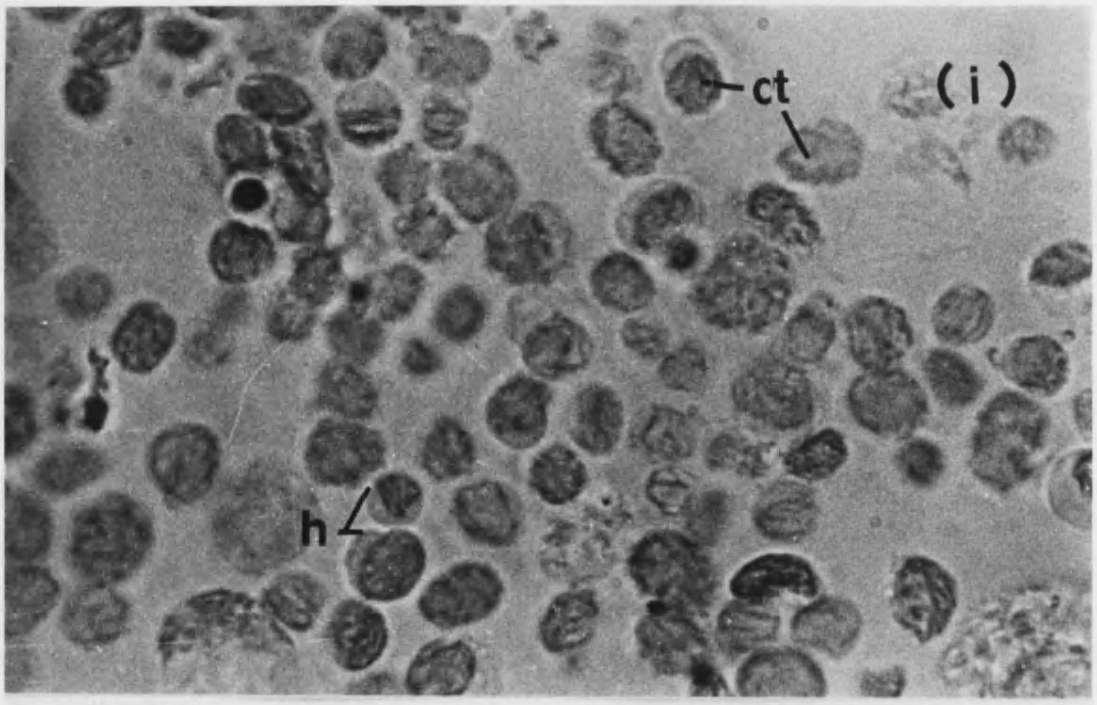
4.3.1 DAPI staining of chloroplasts, protoplasts and amyloplasts

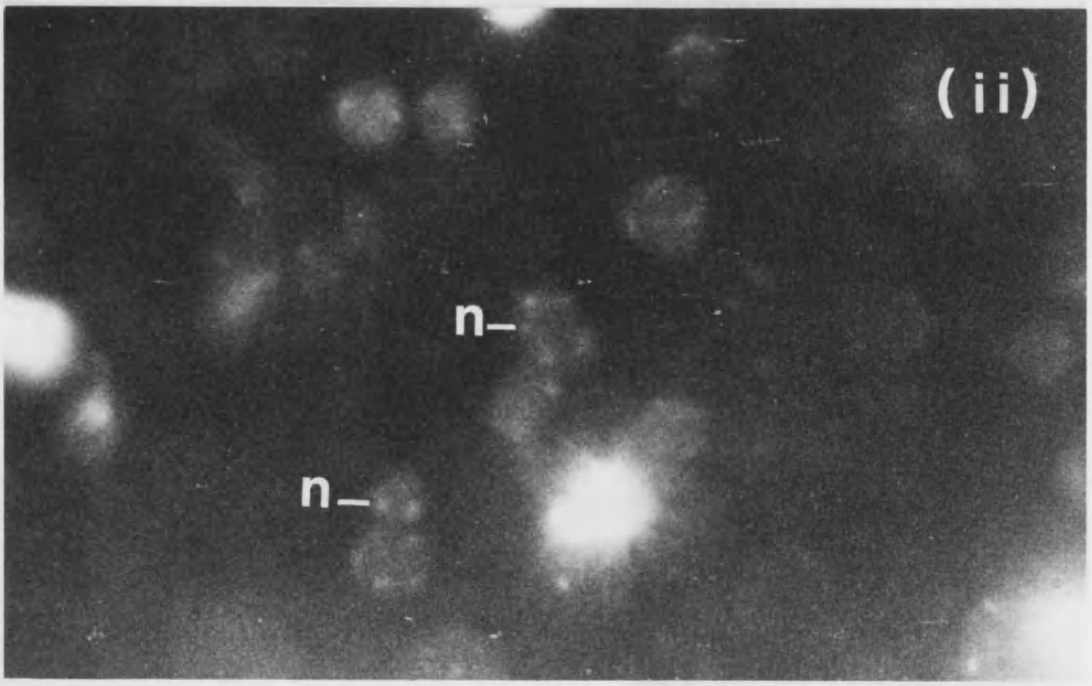
A chloroplast preparation, stained with DAPI and illuminated by tungsten and ultra-violet light, revealed both intact and damaged organelles (Fig. 4.3.1.1(i) and (ii)). Characteristic halos are used to identify those chloroplasts enclosed by an intact membrane bilayer. Herrman (1982) states that unbroken plastids appear refractive with sharp contours and are often cup-shaped. Fragmented chloroplasts are flatter, darker with irregular surfaces and the grana may be visible. Under fluorescent light it was apparent that only intact chloroplasts contained fluorescent nucleoids. At least five nucleoids were visible in most of the organelles.

DAPI stained amyloplasts, unlike chloroplasts, were not separated from cell debris by differential or density gradient centrifugation. As a result, it was unclear whether starch granules which fluoresced did so due to amyloplast DNA contained within the membrane bilayer or because of contamination from extraneous material (Fig. 4.3.1.2(i) and (ii)). It was evident that only the larger starch granules appeared to fluoresce and it was only these larger granules which had well defined halos. Whether these halos should be attributed to intact amyloplasts is not certain since it could be argued that the larger granules simply refract more light. It was observed that the denser the peripheral halo, the more intense the fluorescence in that area.

DAPI stained protoplasts and amyloplasts prepared

Figure 4.3.1.1 Chloroplasts removed from a sucrose gradient and stained in 4 μg/ml DAPI:
(i) under tungsten light, revealing characteristic halos (h);
(ii) the same view under fluorescent light, revealing chloroplast nucleoids (n)
(magnification: 15 μm)


ct
(i)
h


(ii)
n
n

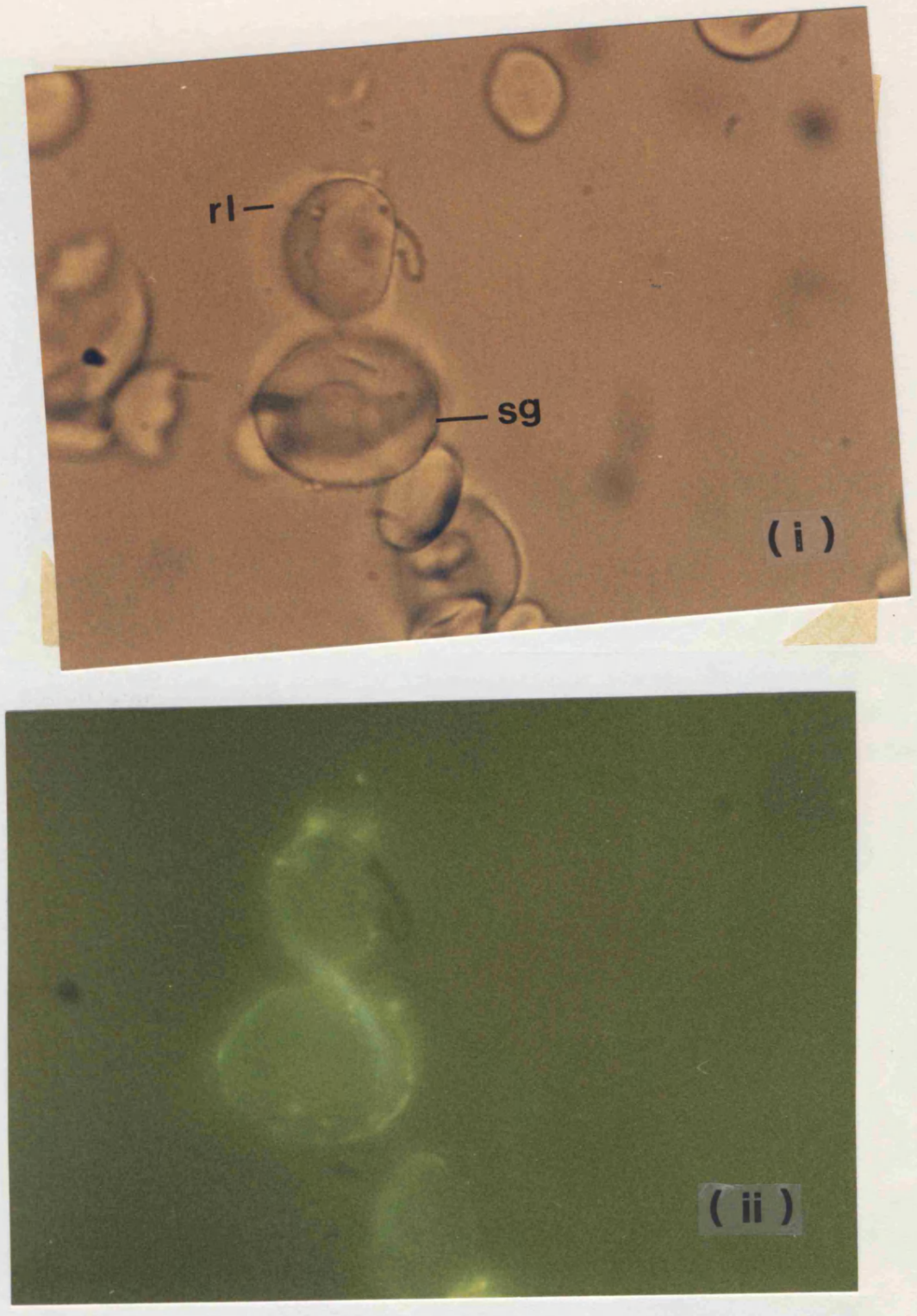
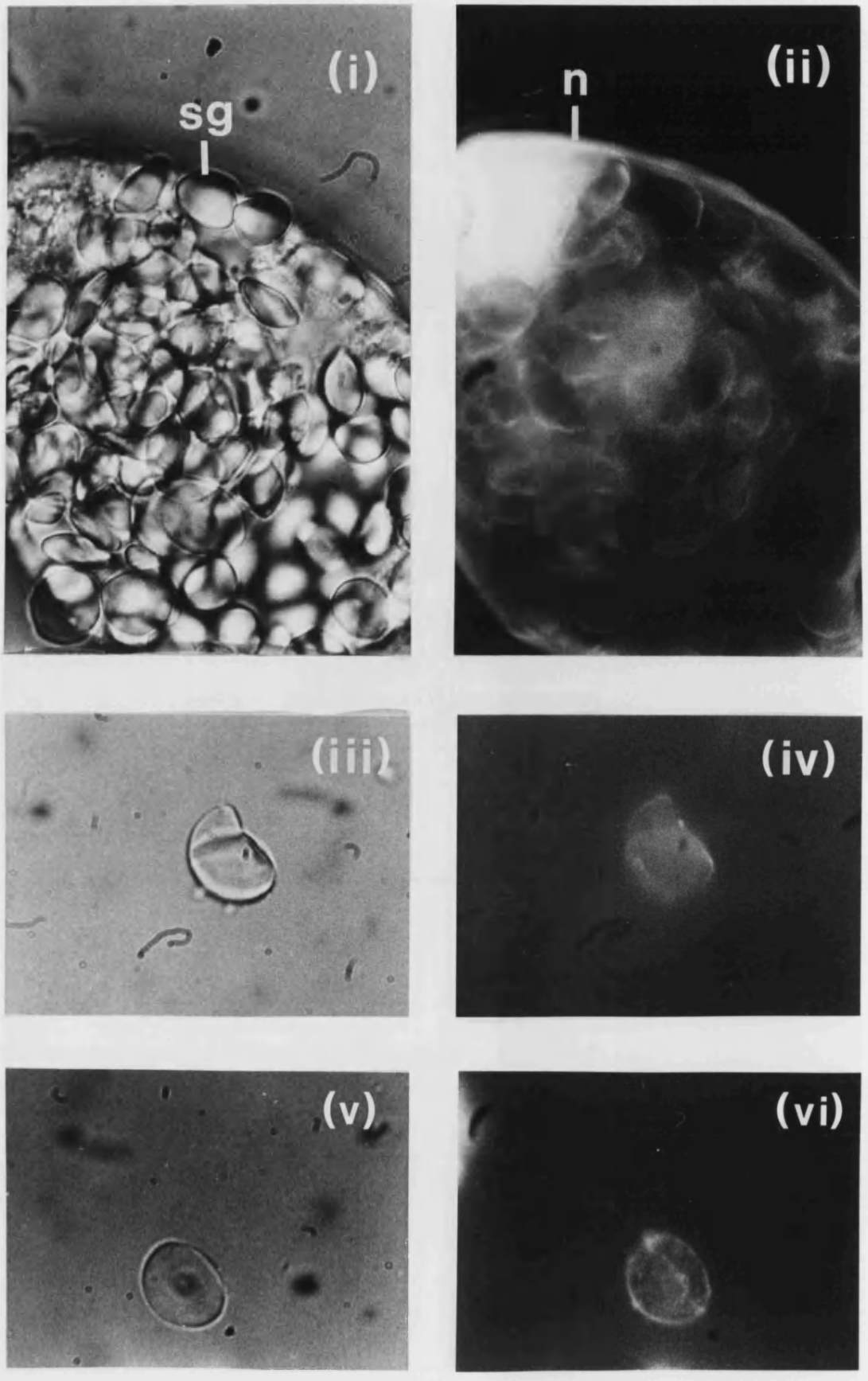


Figure 4.3.1.2 Amyloplasts or starch granules released from protoplasts and stained in 25 μg/ml DAPI:
(i) under tungsten light, revealing refracted light (r.l.) around larger granules;
(ii) the same view under fluorescent light, revealing fluorescence of large granules.
(magnification: 14.4 μm)

Figure 4.3.1.3 Protoplasts (i) and (ii) and starch granules (iii)-(vi) treated with digitonin at 1.1 mg/ml, DNase at 25 μg/ml and DAPI at 25 μg/ml: visualised under tungsten (i), (iii) and (v) and fluorescent (ii), (iv) and (vi) light. (magnification: 41.2 μm)


(i)
sg
(ii)
n
(iii)
(iv)
(v)
(vi)

subsequently were incubated in DNase in an attempt to reduce fluorescence due to any nDNA bound to the amyloplast membranes. Fluorescence of the protoplast nucleus remained intense (Fig. 4.3.1.3(i) and (ii)): clearly the dye, but not the DNase, was able to penetrate the cell membrane and, assumably, the amyloplast membranes, under these conditions. The DNase treatment did facilitate viewing of the amyloplast preparations but probably a higher concentration of DNase or more extended incubation would have improved results further still. Starch granules were less contaminated but the more general fluorescence all over the granule surface was possibly undiminished (Fig. 4.3.1.3(iii)-(vi)). Whether the patches of more intense fluorescence were due to aggregated DNA within or external to the amyloplast is uncertain. Although it is quite likely that halos are only visible where the membranes are intact, it cannot be assumed that the fluorescence is wholly due to amyloplast DNA.

Digitonin treatment of protoplasts may have increased protoplast lysis, although this was uncertain. Under the conditions used here, neither protoplasts nor amyloplasts appeared to be severely affected.

4.3.2 Southern blots probed with P6 and P7

Restriction of total endosperm and leaf DNA with PstI, which is inhibited by methylation of the adenine residue in the recognition sequence CTGCAG (Kessler and Höltke, 1986), did not appear to significantly affect DNA size, since restricted and non restricted DNA samples appeared to

comigrate (Fig. 4.3.2.2(i)). This indicated that the heavily methylated genomic DNA (Herrman, 1972) was not restricted much by PstI.

P7, an 8.1 kb ctDNA fragment which includes atpE and part of atpB (Fig. 4.3.2.1), hybridised very strongly to Southern blots of total leaf DNA and less well to total endosperm DNA (Fig. 4.3.2.2(ii)). The low level of hybridisation to total endosperm DNA implicated that there is ptDNA in endosperm tissue, but in considerably lower amounts than in leaf. That this low level of hybridisation is to ptDNA, rather than background hybridisation to homologous sequences in nDNA or mitochondrial DNA (mtDNA), is probable since an 8.1 kb fragment (the size of P7) was visible in Southern blots of both leaf and endosperm DNA after PstI digestion. In fact background hybridisation to DNA of more than this size was quite low.

Similar results were obtained using the ctDNA fragment P6 (Fig. 4.3.2.3), an 8.4 kb fragment within the inverted repeat region of the plastome, including the 16S ribosomal RNA (rRNA) gene (Fig. 4.3.2.1). Background levels of hybridisation were again quite low, and hybridisation to endosperm DNA was more evident, confirming the previous conclusion.

Hybridisation to DNA in Southern blots is very dependent on the state of the DNA, badly degraded samples hybridising less well. It is possible that the relatively low level of hybridisation of total endosperm DNA to P7, compared with P6, was not due to different amounts of these in amyloplast

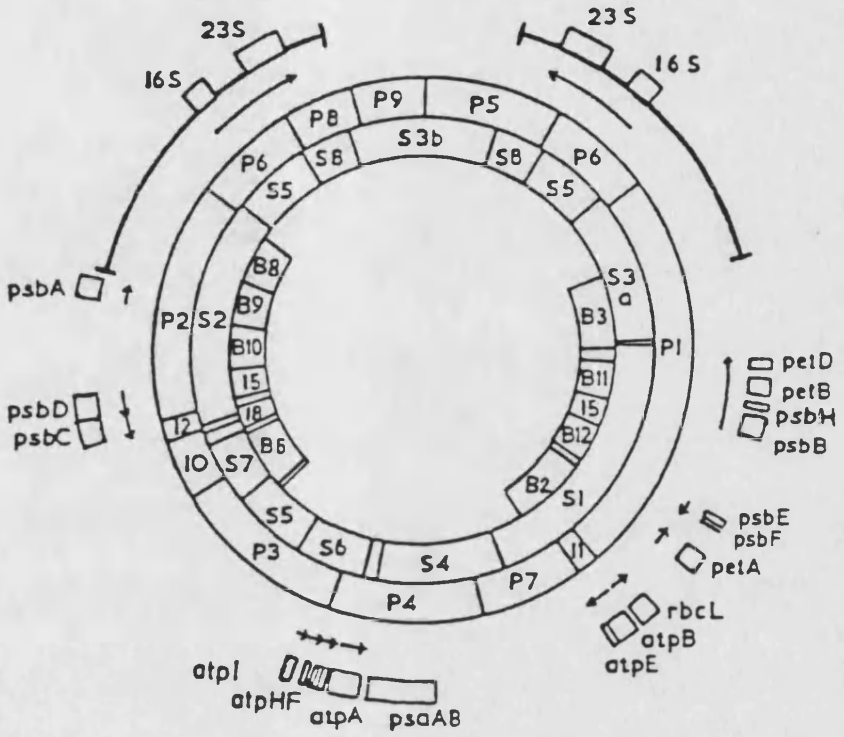


Figure 4.3.2.1 Restriction map of wheat ctDNA showing PstI, SalI and BamHI recognition sites (based on Bowman et al., 1981; Courtice et al., 1985; Hird et al., 1986b). Position of the genes for the 16S and 23S rRNAs (Bowman et al., 1981), the large subunit of ribulose bisphosphate carboxylase (rbcL, Bowman et al., 1981), the α,β,ε, I and III subunits of ATP synthase (atpA,B,E,F and H; Hird et al., 1986a and atpI; Hird et al., 1986b), cytochrome f, cytochrome b-563 and the 15 kDa polypeptide of the cytochrome complex (petA, B and D; Hird et al., 1986a), the 32 kDa, 51 kDa, 44 kDa, D2, 9 kDa (cytochrome b-559), 4 kDa and 10 kDa polypeptides of photosystem II (psbA,B,C,D,E, F and H; Courtice et al., 1985; Hird et al., 1986a,b) and the 60 kDa polypeptide of photosystem I (included in psaAB; Hird et al., 1986b). For location of tRNA genes see Quigley et al. (1985); Quigley and Weil (1985) and Mubumbila et al. (1985).

Figure 4.3.2.2 Southern blot of total leaf and endosperm nucleic acids probed with P7

(i) Agarose gel loaded with:

1. 4 μg total leaf nucleic acid
2. 4 μg PstI restricted total leaf nucleic acid
3. 8 μg PstI restricted total leaf nucleic acid
4. 4 μg total endosperm nucleic acid (approx. 20 d.p.a.)
5. 4 μg PstI restricted total endosperm nucleic acid
6. 8 μg PstI restricted total endosperm nucleic acid

(ii) Southern blot of the same gel

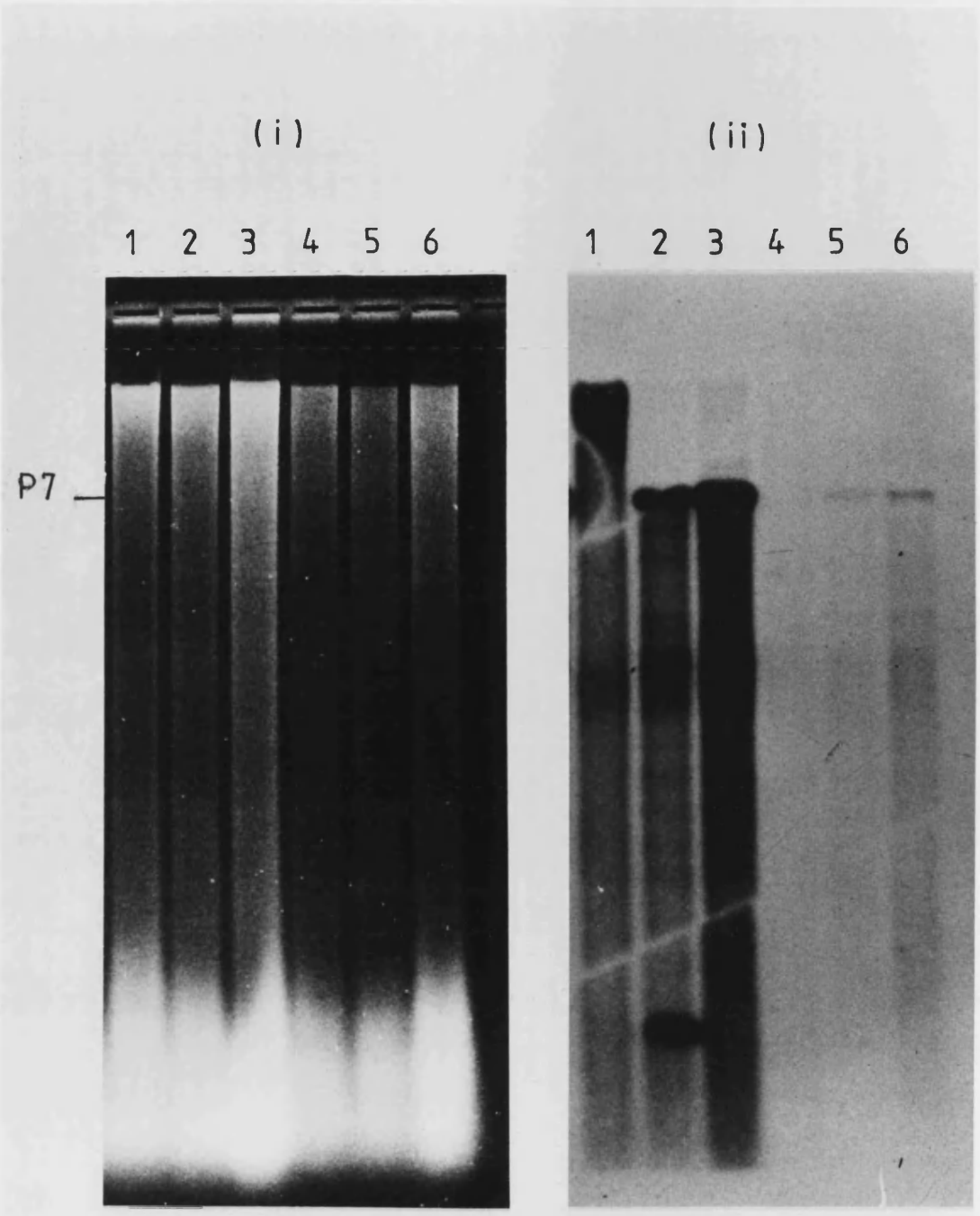

(i)
(ii)
1 2 3 4 5 6
1 2 3 4 5 6
P7

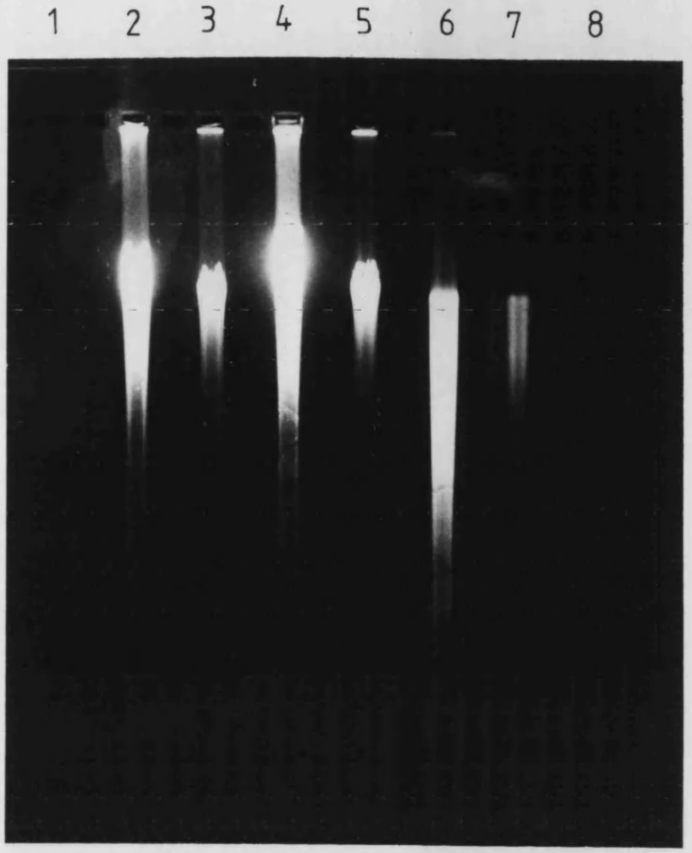
Figure 4.3.2.3 Southern blot of total leaf and endosperm DNA probed with P6

(i) Agarose gel loaded with:

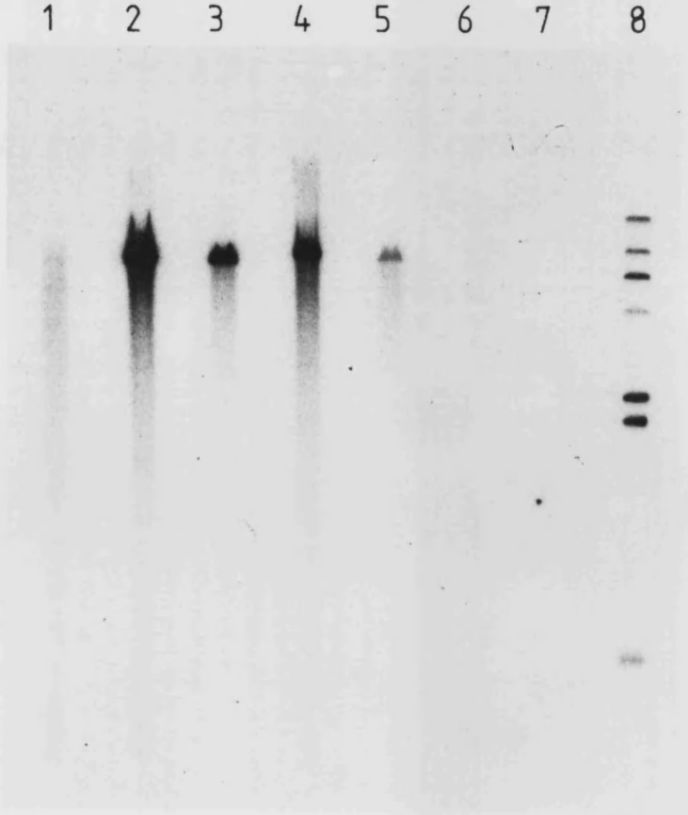
1. ctDNA
2. 2.5 μg PstI restricted total leaf DNA
3. 0.5 μg PstI restricted total leaf DNA
4. 5.4 μg PstI restricted total endosperm DNA (13 d.p.a. C grains)
5. 1.1 μg PstI restricted total endosperm DNA (13 d.p.a. C grains)
6. 3.3 μg PstI restricted total endosperm DNA (26 d.p.a. A grains)
7. 0.2 μg PstI restricted total endosperm DNA (26 d.p.a. A grains)
8. Radiolabelled HindIII digested λ DNA

(ii) Southern blot of the same gel.

(i)



(ii)



ptDNA, rather it may have been because the DNA hybridised with P7 was quite degraded.

From these results it was concluded that either P7 or P6 should be a suitable probe for ptDNA, since background hybridisation to the unrestricted nDNA appeared to constitute only a fraction of the total.

4.3.3 Reassociations

Reassociation kinetics data are presented as Cot curves, as described by Chelm (1982) and Britten and Kohne (1968), or as $Cot\frac{1}{2}$ values (Steele Scott *et al.*, 1984) (see Appendix IV). The following equation was used to estimate percentage ptDNA:

$$\frac{Cot\frac{1}{2} \text{ ptDNA standard}}{Cot\frac{1}{2} \text{ total leaf or endosperm DNA}} \times 100$$

since the time elapsed before half renaturation of the ptDNA probe with a total DNA sample is directly proportional to the concentration of complementary sequences in the driver DNA, as long as they are in sufficient excess over the probe.

Size of probe DNA

Before commencing with reassociations reactions it was necessary to obtain DNA samples of relatively uniform fragment sizes since it has been found that the rate of reannealing is proportional to the square root of DNA length (Young and Anderson, 1985). Britten and Kohne (1968) suggest that fragments of 400 to 500 bp should give reproducible

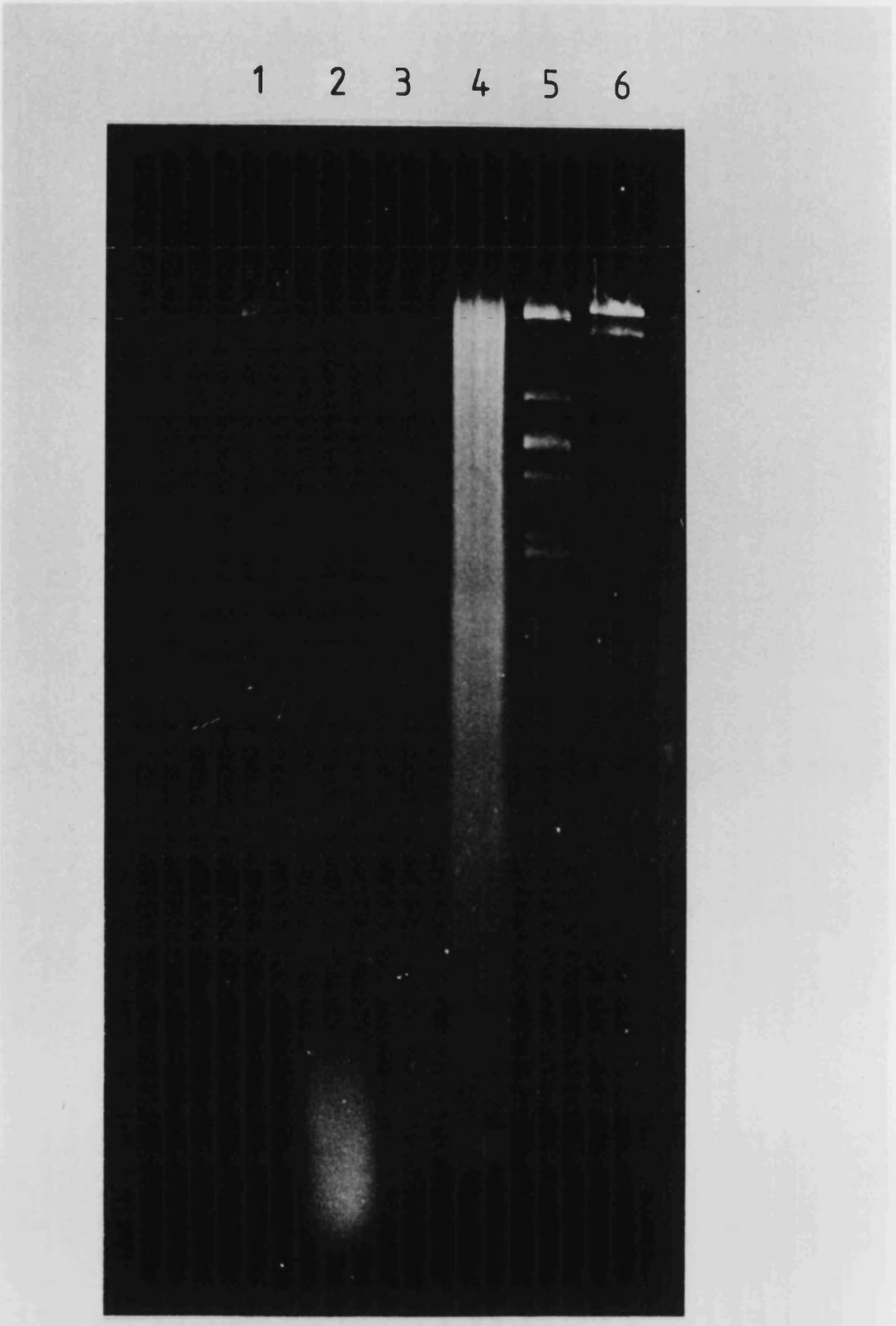
reassociation rates and do not tend to form large aggregates and networks.

In order to obtain a uniform probe length, sonication conditions were tested (always at 35 watts) and DNA size was estimated by agarose gel electrophoresis. First, total leaf DNA was sonicated for 8 min using a 1 cm diameter probe immersed in an icy waterbath, in which an eppendorf containing the DNA was suspended. However, the DNA was little affected, electrophoresing at a molecular weight of more than 10 kb.

When nick translated [^{3}H]ctDNA, was sonicated for 4 and 8 min, using a 3 mm diameter probe, the resultant fragments were too small: 8 min sonicated DNA ran ahead of the bromophenol blue marker and the molecular weight of 4 min sonicated DNA was considerably less than 400 bp. Non-sonicated [^{3}H]ctDNA covered a wide size range of more than 40 kb to less than 1 kb. However, ctDNA sonicated for 3.5 min, with a 3 mm probe, spanned from 230 to 500 bp, with peak intensity at around 290 bp, and ctDNA sonicated for 2.5 min spanned from 300 to 700 bp (Fig. 4.3.3.1, lanes 2 and 3), with most of the DNA electrophoresing at around 400 bp. CtDNA which had been sonicated for 2.5 min, with a 3.0 mm probe, and then nick translated with ^{3}H[TTP] electrophoresed slightly ahead of the cold 2.5 min sonicated ctDNA.

From these data it was determied that, for reassociations, the DNA would be sonicated for 2.5 min, at 35 watts, using a 3.5 mm diameter probe (cf. Ersland et al., 1981). As far as it was possible, sonicator noise was also

Figure 4.3.3.1 Agarose gel of sonicated and restricted ctDNA:
1. EcoRI digested lambda DNA
2. 3.5 min sonicated ctDNA
3 2.5 min sonicated ctDNA
4. PstI digested ctDNA
5. EcoRI and BamHI digested lambda DNA
6. SalI digested lambda DNA.


1
2
3
4
5
6

kept constant.

Results of ptDNA reassociations

The first reassociations carried out gave very variable estimates of the ptDNA content of endosperm and leaf tissues, therefore attempts were made to improve the assay procedure so that results might be more reproducible (see Appendices II and III). Using improved assay conditions it became apparent that the ptDNA probe was reassociating extremely slowly, with $Cot\frac{1}{2}$ values of 60 for total leaf DNA and more than 1000 for total endosperm DNA (Table 4.3.3). These figures were considerably greater than the published data for similar tissue types and conditions (Steele Scott et al., 1984). In the Cot curves presented (Figure 4.3.3.2(i)) the total endosperm ptDNA reassociated too slowly for the $Cot\frac{1}{2}$ to be accurately determined. It also appeared that the DNA was not reassociating as a simple, second order reaction typical of a single component reassociation, where the reassociation should take place over two log units of Cot (Britten and Kohne, 1968), but appeared to be multicomponent. It was considered probable that this was due to nDNA contamination of the [^{3}H]ctDNA probe, the repeat sequences in the nDNA possibly reassociating before or after the ctDNA reassociation, depending on the degree of contamination.

At this point a series of attempts were made to improve ctDNA purity, prior to carrying out further reassociations. These measures are described in the next section (4.3.4). The resultant ctDNA appeared to give considerably better

Table 4.3.3 $Cot\frac{1}{2}$ estimates from reassociations using [^{3}H]ctDNA probes

Probe DNA	Driver DNA	$Cot\frac{1}{2}$ (mol.l^{-1}.s)
Original probe	Chloroplast DNA	18.0-39.8
	Total leaf DNA	59.6
	Total endosperm DNA:	
	C grains at 27 d.p.a.	2754
	C " " 45 "	145
	C " " 36 " (intact spikes)	1000
	C " " 36 " (degrained spikes)	4786
	C " " 27 "	1047
	C " " 36 " (degrained spikes)	891
New probe	Chloroplast DNA: lower band	0.81-1.74
	mixed band	5.43-13.8
	upper band	125.9
	Total leaf DNA	1.91
	Total endosperm DNA:	
	A grains at 9 d.p.a.	15.9
	A " " 13 "	20.9
	A " " 17 "	8.7
	A " " 21 "	50.1
	A " " 26 "	125.9
	C " " 13 "	3.16
	C " " 60 " (intact spikes	12.6
	C " " 60 " (degrained spikes)	36.3

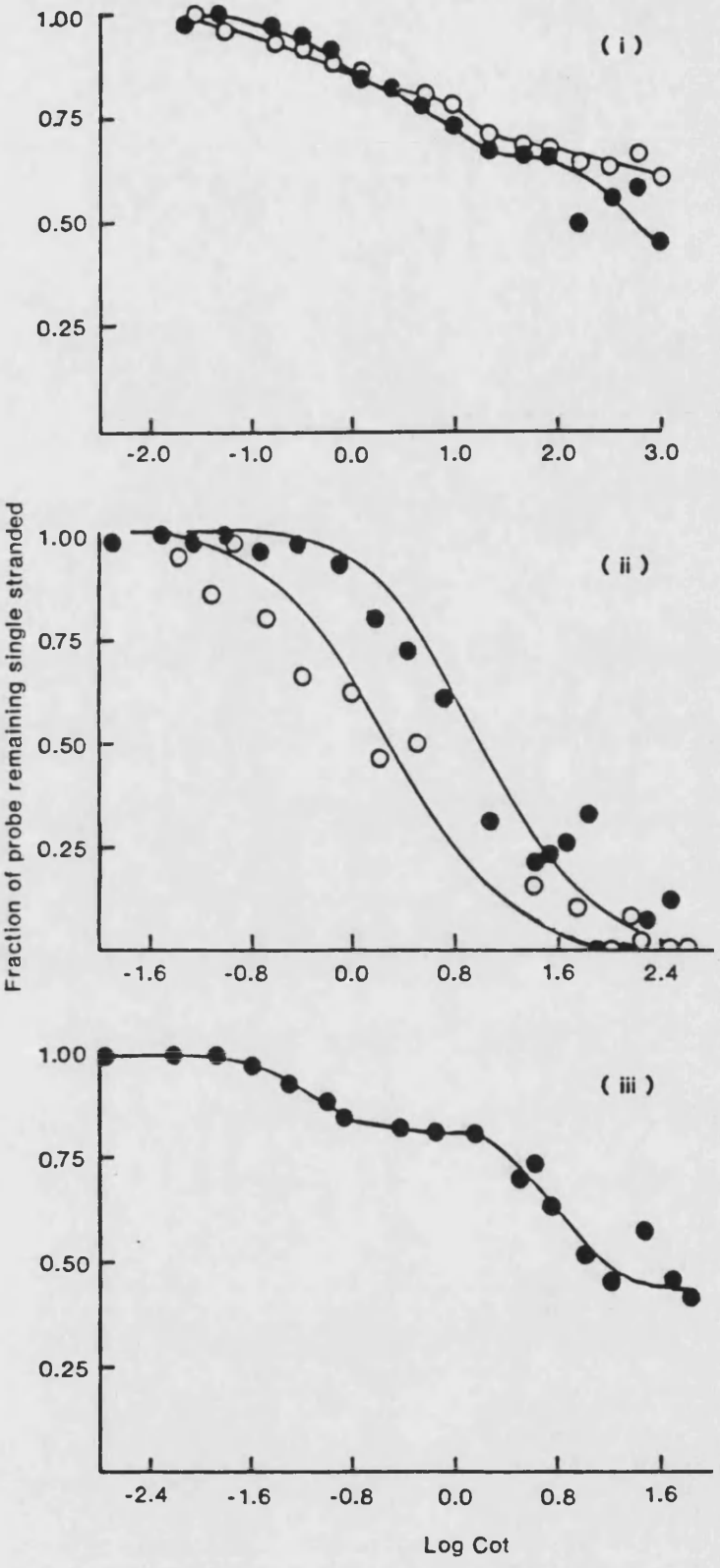


Figure 4.3.3.2 Cot curves: (i) original ctDNA probe reassociated with total endosperm DNA from C grains at 36 d.p.a., from intact (●) and degrained (○) spikes, (ii) new ctDNA probe reassociated with total leaf DNA (○) and total endosperm DNA from A grains at 17 d.p.a. (●), showing ideal curves, and (iii) new ctDNA probe reassociated with total endosperm DNA from A grains at 21 d.p.a.

results: the reactions were more rapid, going to completion, and more closely fitted the ideal curve so that it was not immediately evident that there was more than one component present (Fig. 4.3.3.2(ii)). However, reassociations using more mature endosperm tissue which reassociated less rapidly, indicated quite clearly that there were at least two components reassociating and that there was more than a two-fold difference between the rates (Fig. 4.3.3.2(iii)). Since it was not possible to distinguish which of the rates could be attributed to ctDNA reassociation, the ptDNA rate constants for the different tissues could not be estimated.

When $Cot\frac{1}{2}$ values were estimated for the whole curve, rather than the individual components (Table 4.3.3), it was found that the results showed a trend towards an increase in value for endosperm tissue of increasing age (which would indicate that percentage ptDNA was declining). It was considered that possibly not all of the starch had been extracted from the endosperm DNA preparations and that, in some way, this may have impeded the rates of DNA nucleation. Conceivably, even with a pure ctDNA probe, or a cloned ctDNA fragment probe, the rates of reassociation in solution may not accurately represent the true ptDNA concentration.

Because of both of these problems, the reassociations were not continued but dot blots were used instead in order to estimate the percentage ptDNA in endosperm.

4.3.4 Chloroplast DNA purity

Since initial reassociation experiments had indicated

that ctDNA preparations were contaminated with other DNA, measures were taken to try to prevent this. The following modifications to the original procedure were adopted, as suggested by Herrmann (1982): (i) young leaf tissue was harvested at 7-14 days after germination and the yield of intact chloroplasts compared with that from eight week old leaves; (ii) 0.05% w/v BSA and 3 mM EDTA were included in the homogenisation buffer as well as 0.04 M mercaptoethanol: $MgCl_2$ was omitted; (iii) the buffer was semifrozen and plant tissue washed in sterile distilled water at 4°C before homogenising; (iv) a pelleting spin at 1500 x g for 1 min, originally included before differential centrifugation, was omitted as intact chloroplasts were also pelleted; (v) chloroplasts were pelleted at 3000 x g for 2 min and then repelleted at 3000 x g for 1 min, rather than the previous 10 min pelleting spins; (vi) a DNase treatment was carried out to remove extraneous DNA: recovered chloroplasts were incubated at 4°C for 30 min in 50 μg/ml DNase; (vii) chloroplasts recovered from sucrose gradients were treated with 100 μg/ml predigested protease and 50 μg/ml RNase, at 37°C for 4 h, in an attempt to improve chloroplast lysis and the yield of ccctDNA molecules and (viii), alternatively, chloroplasts were lysed in 20 mM NaCl, 70 mM EDTA and 3% w/v Sarcosyl.

Sucrose gradients of chloroplasts extracted from leaves after 7-14 days and eight weeks of growth were compared (Fig. 4.3.4.1). Rocha and Ting (1970) claim that intact chloroplasts have a higher isopycnic density and sediment

more rapidly than broken chloroplasts. On this basis it would appear that all of the chloroplasts from the younger leaves were damaged, since the lower band was scarcely visible, whereas the older tissue yielded two bands of chloroplasts. However, light microscopy of chloroplasts from sucrose gradients indicated that in the upper bands a large number of chloroplasts appeared to be intact (Fig. 4.3.4.2(v) and section 4.3.1).

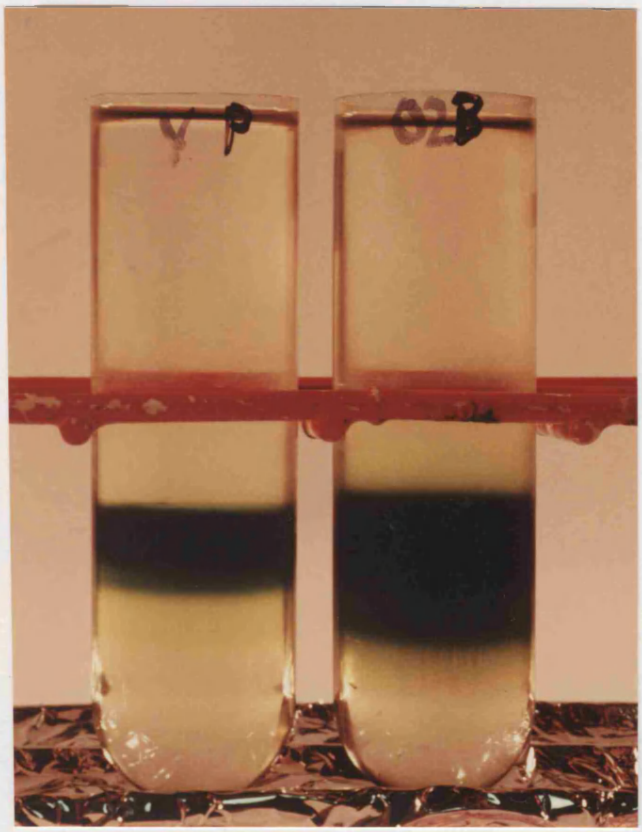
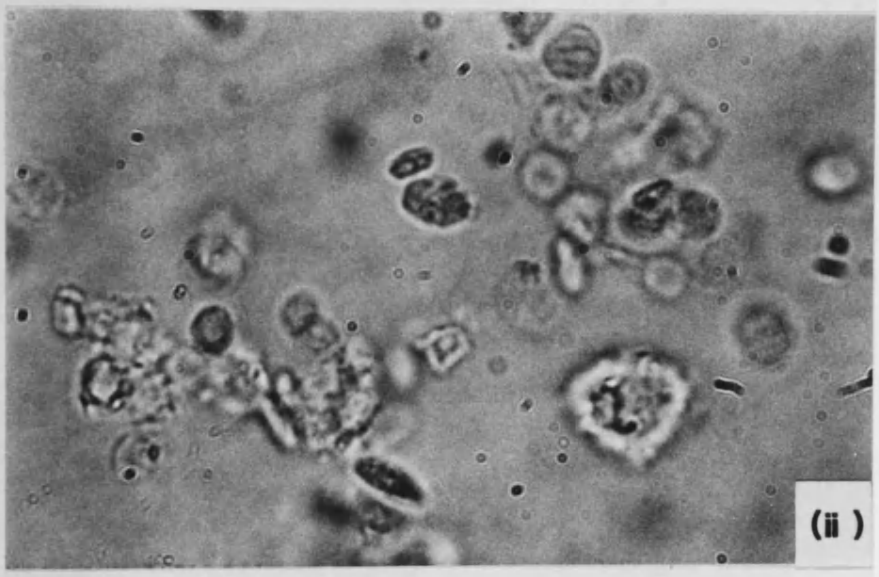


Figure 4.3.4.1 Sucrose gradients of chloroplasts extracted from 7-14 day old (left) and 8 week old (right) wheat leaves.

Figure 4.3.4.2 Light and fluorescence micrographs of DAPI stained wheat chloroplasts from sucrose gradients:
(i) fluorescence of chloroplasts before sucrose gradient centrifugation
(ii) chloroplasts from the lower band on a sucrose gradient
(iii) fluorescence of the same chloroplasts
(iv) protoplasts from the lower chloroplast band of another gradient
(v) intact chloroplasts from the upper band of a sucrose gradient
(vi) chloroplasts from the upper band of another gradient
((ii), (iii) and (iv) were prepared from eight week old plants, (i), (iv) and (vi) from a mixture of two week and more mature leaves)
(magnification: 15 μm)


(i)


(ii)

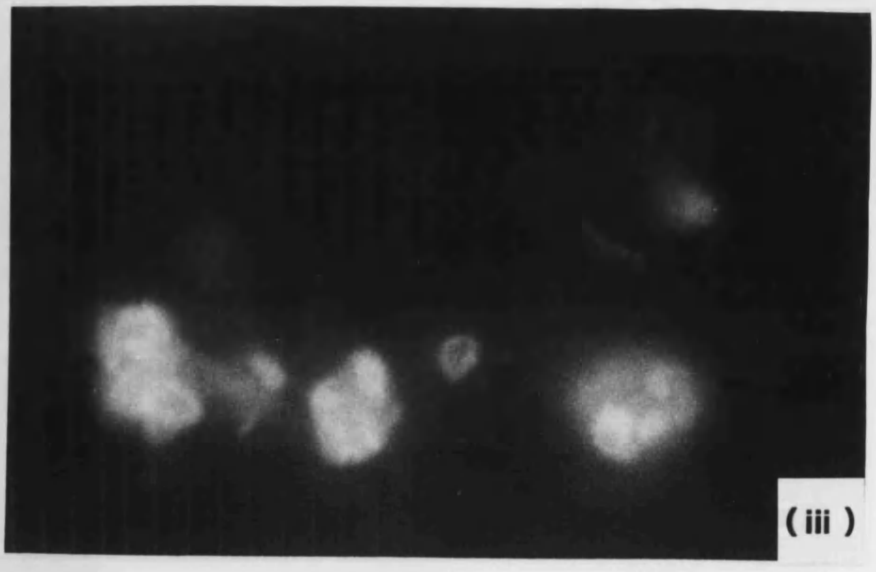
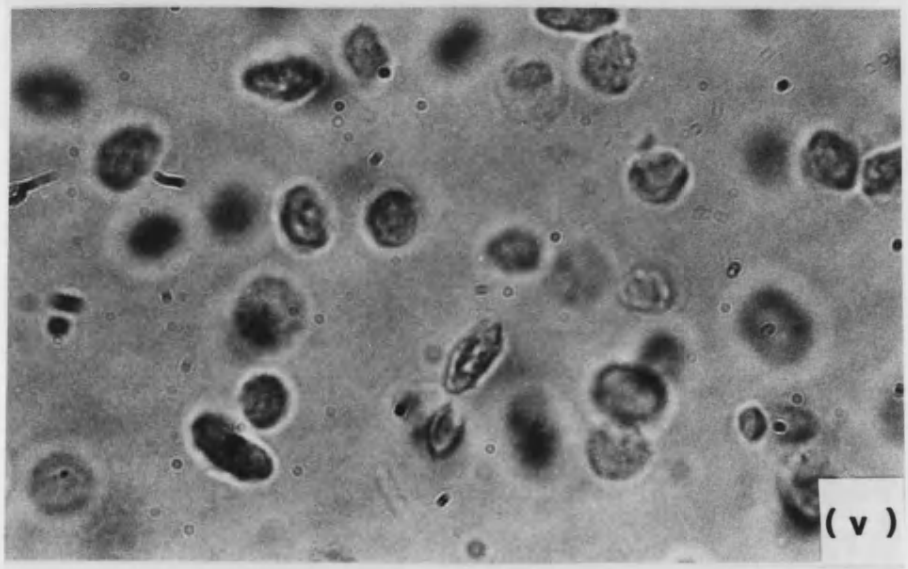
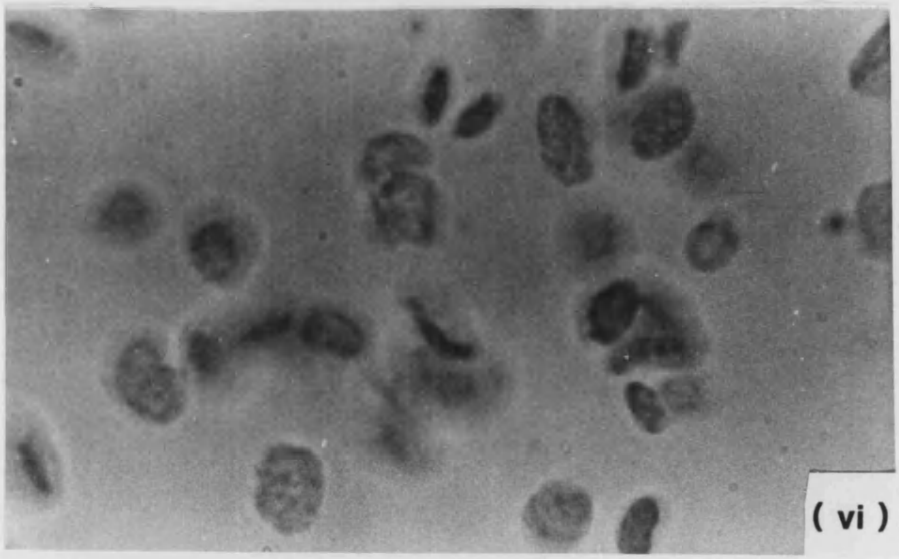

(iii)

Figure 4.3.4.2 cont'd. Light and fluorescence micrographs of DAPI stained wheat chloroplasts from sucrose gradients.

(iv)



(v)



(vi)

DAPI stained chloroplasts applied to sucrose gradients were heavily contaminated with fluorescent material (Fig. 4.3.4.2(i)), presumably extraneous DNA and nuclei since DAPI is highly DNA specific (James and Jope, 1978). Chloroplasts from the lower sucrose band were largely intact but contaminated with nuclei, protoplasts and other debris (Fig. 4.3.4.2(ii), (iii) and (iv)). In comparison chloroplasts from the upper band were considerably less contaminated (Fig. 4.3.1.2(v) and (vi)) and for two out of three extractions there appeared to be a high proportion of chloroplasts which appeared intact in this band. Damaged chloroplasts prepared from the upper band are shown (Fig. 4.3.4.2(vi)). Sucrose gradients of chloroplasts pelleted for 5 min at 3000 x g revealed greater levels of contamination than those pelleted for 1-2 min.

Both the protease and RNase and the DNase treatments, carried out on separate extractions, failed to yield any DNA on CsCl gradients. The use of 20 mM NaCl and 70 mM EDTA in the chloroplast lysis buffer was also not profitable: the addition of CsCl to this mixture caused the chloroplasts to clump, further 35% v/v Sarcosyl was added to overcome this. In addition, dense chloroplasts banding at the top of CsCl gradients occasionally caused the gradients to collapse. In a subsequent ctDNA preparation, lysis of chloroplasts in 1 or 2% v/v Sarcosyl was shown to be adequate, yielding bands of ccctDNA on CsCl gradients. Chloroplasts lysed with 4% v/v Sarcosyl from another extraction only yielded linear DNA.

On several occasions ccctDNA was prepared from

chloroplasts taken from the upper band on sucrose gradients. This may be either because there were intact or, possibly, permeabilised chloroplasts in this band, or because ctDNA can be extracted from broken chloroplasts.

Not all DNA extractions yielded two bands on CsCl gradients, where there was only one band it was assumed to contain linear ctDNA and any nDNA contamination. High yields (more than 5 μg) of ccctDNA, in the lower CsCl band, were never obtained.

4.3.5 Preliminary dot blots

The first dot blots of total leaf and endosperm DNA samples, which were probed with pTacP6 (pBR322 containing P6), indicated that the average ptDNA content of endosperm DNA was approximately 8% of that for leaf DNA, but it was not possible to determine absolute figures.

For the following dot blots, using a slot blot apparatus, triplicate filters were probed with P6 and B2 and the plasmid pTA71. These dot blots, as well as yielding results, helped to determine the best approach, necessary controls and how to minimise error.

Linearity of probe hybridisation to filter bound DNA

For the slot blots presented (Tables 4.3.5.2 and 4.3.5.3) it was found that probe hybridisation to filter bound sequences up to 0.4 ng showed a linear relationship suggesting that hybridisation was proportional to the concentration of filter bound complementary sequences (Fig.

4.3.5.1(i) and (ii)). For loadings of 0.4 to 1.0 ng the rate of hybridisation, instead of decreasing as would be expected at higher loadings if the concentration of filter bound probe becomes much greater than that in solution (Anderson and Young, 1985)(see Appendix IV), appeared to increase. Subsequent data for loadings of P6 up to 7.5 ng and B2 up to 4.3 ng did not show the same increase: it was therefore concluded that the data in Fig. 4.3.5.1 represent a linear increase up to 1.0 ng. All dot blots contained probe concentrations of 20 ng/ml of Hybridisation buffer and standard curves using probe dots up to 3.5 ng or more were linear. Dots of up to 0.5 μg of total leaf DNA fell within the linear range of these standard curves.

Comparison of methods of detection of hybridised probe

The curves of probe detected by both Cerenkov and liquid scintillation counting show the same trends (Fig. 4.3.5.1(i) and (ii)), although the liquid scintillation counts were twice as high. Since scintillation counting was more sensitive all subsequent dot blots were counted in scintillant.

Autoradiographs of filters when scanned with a densitometer gave a very different standard curve, the linear relationship between the extent of hybridisation and amount loaded did not extend far (for the autoradiograph used here it was only to 0.15 ng of P6) (Fig. 4.3.5.1(iii)). This was because the linear response of the X-ray film was saturated at higher film intensities, since film density is only

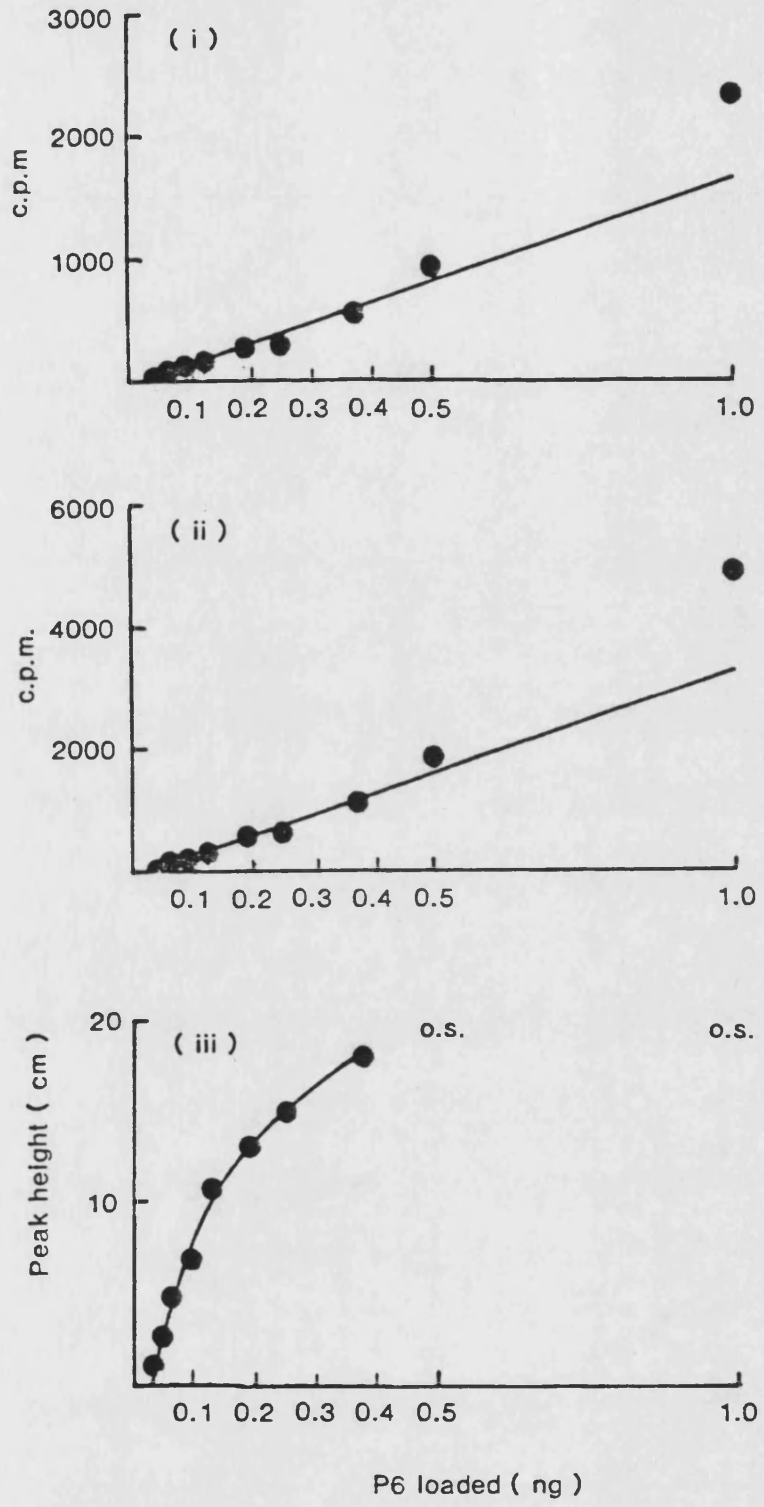


Figure 4.3.5.1 Comparison of methods for detecting hybridised probe:

(i) Cerenkov counting

(ii) Liquid scintillation counting

(iii) Peak height of densitometric scan of autoradiograph

(o.s. = off scale)

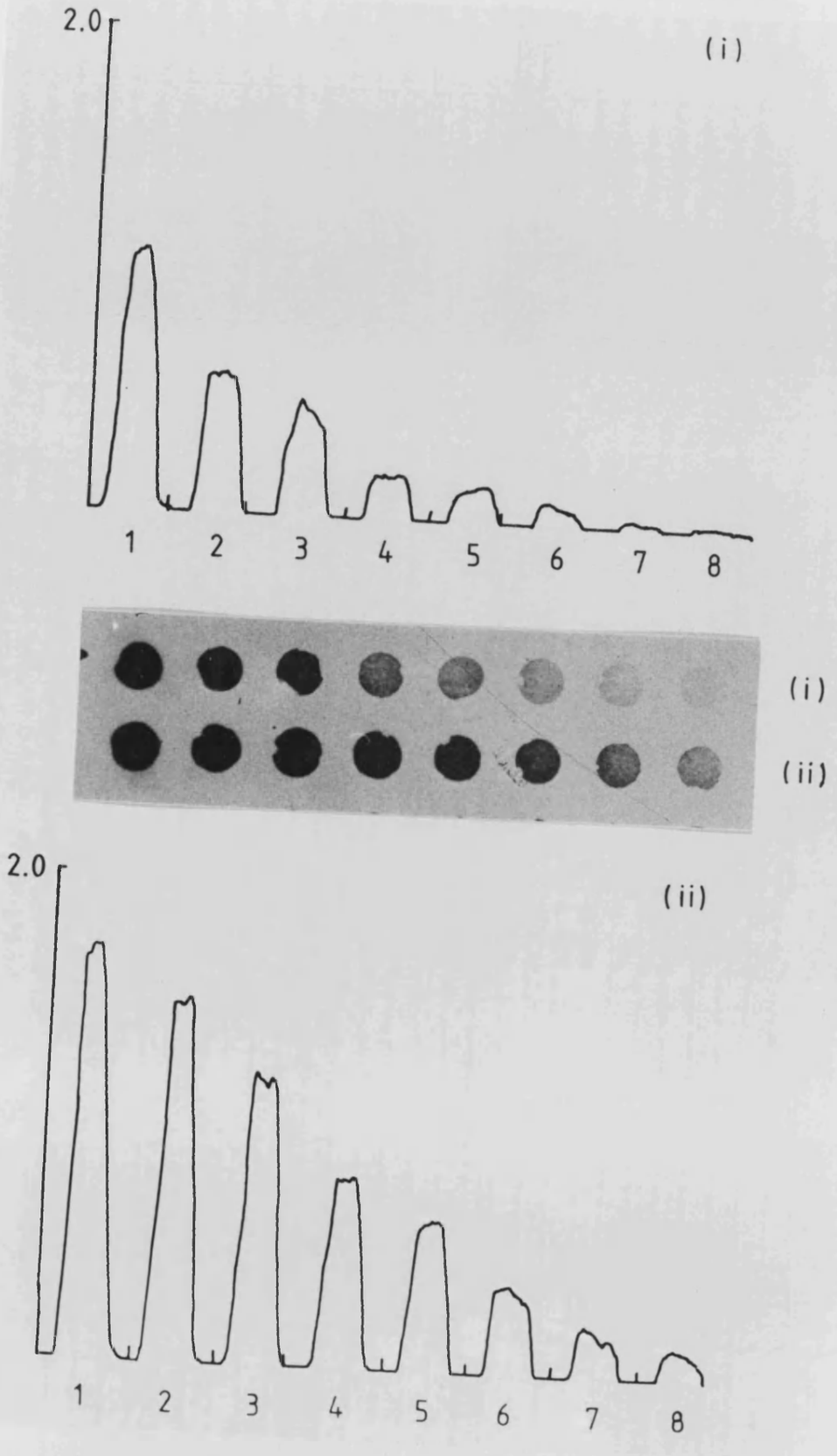
directly proportional to exposure to β particles when density is low (Laskey and Mills, 1975). Intensity is a function of exposure and developing time as well as the amount of radiolabel; therefore it is possible to extend the linearity, but not without a loss of sensitivity. In practice, it was not simple: the amounts of complementary sequences loaded needed to fall within a narrow range with less than a four fold variation for accurate measurement. This can be seen from the scans presented in Fig. 4.3.5.2(i) and (ii) which are either insufficiently sensitive or not linear, making standardisation and comparison of samples difficult.

However, on occasions when the hybridised counts were too low to be accurately detected by scintillation counting, densitometric scans of autoradiographs were used to obtain results. Since autoradiographs can be exposed for a number of weeks this technique has the potential of being considerably more sensitive.

CtDNA as a probe

That wheat ctDNA, when extracted as described, could not be used as a probe for ptDNA in leaf or endosperm DNA was highlighted by two experiments. In the first, samples of ctDNA were prepared from the upper and lower bands and a mixed band of DNA from CsCl gradients of chloroplasts (from sucrose gradients). These samples were loaded on slot blots probed with P6, B2 and pTA71 and the percentage ptDNA in each was estimated. Results (Table 4.3.5.1) showed that all three samples failed to hybridise to P6 and B2 as much as expected

Figure 4.3.5.2 Absorbance scans of a dot blot autoradiograph:
(i) total leaf DNA: loadings are doublings from 4.0 ng (8)to 500 ng (1)
(ii) probe DNA (P6): loadings are doublings from 0.034 ng (8) to 4.35 ng (1)


2.0
(i)
1
2
3
4
5
6
7
8
(i)
(ii)
2.0
(ii)
1
2
3
4
5
6
7
8

for pure ctDNA preparations (100%).

Table 4.3.5.1 Estimated percentage ptDNA in ctDNA preparations from CsCl gradients

	Probe	% ptDNA		$\bar{x}$	(σ_{n-1})
Upper band	P6	3.69	3.69	3.10	(0.83)
	B2	1.93	3.08		
Lower band	P6	23.76	8.17	16.52	(8.3)
	B2	23.58	10.65		
Mixed band	P6	8.90	10.58	10.73	(1.3)
	B2	11.90	11.53		

Each estimate is the mean for one filter of four dots.

The upper band, which consists of either linear ctDNA or other linear DNA molecules from the chloroplast preparation, appeared to contain very little ctDNA, possibly as little as 3.1%. The lower band which was expected to consist of only ccctDNA (Vedel and Quetier, 1978; Kolodner and Tewari, 1975) appeared to contain only 16.5% ptDNA. Since the ctDNA samples were not assayed by diphenylamine, these estimates may be low due to starch or some other contamination. Despite this, it is likely that there was considerable contamination of the samples with DNA from other cell compartments, either mitochondrial or nuclei. The nuclear ribosomal probe, pTA71, hybridised to all three samples; crude estimates indicated 23% nDNA contamination of the upper band and 52% nDNA contamination of the lower band.

In the second experiment duplicate dot blots of total endosperm and leaf DNA samples were probed with either ctDNA or P6 (Fig. 4.3.5.3(i) and (ii)). CtDNA hybridised poorly to itself ((i) lanes 3 and 9), indicating that the concentration spotted was probably less than expected, and weakly to P6 ((i) 12 (A-D) and 6 (E-H)), indicating that there were few homologous sequences in the ctDNA probe. P6 hybridised fairly strongly to ctDNA ((ii) 12 (A-D) and 6 (E-H), but indicated an approximate ptDNA content of only 25%. The clearest indication of ctDNA contamination was drawn by comparison of the levels of hybridisation of total leaf DNA samples with total endosperm samples. For the filter probed with ctDNA the leaf samples (10 (A-D) and 5 (E-H)) appeared to hybridise at most twice as strongly as the endosperm samples (1,2,4-8 (A-D), 1,2,7,8,10-12 (E-H)) whereas the filter probed with P6 showed a four to eight fold difference. From this it would appear that the contaminated ctDNA probe was hybridising predominantly to DNA other than ptDNA.

Neither ctDNA nor P6 hybridised significantly to the herring sperm DNA control (11 (A-D)).

Comparison of methods for determining percentage ptDNA

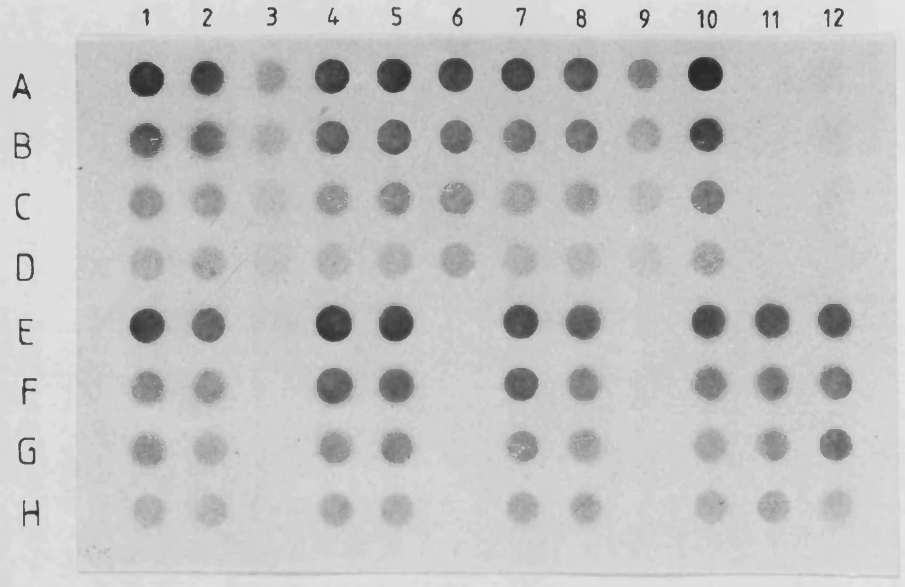
Since the plasmid wheat ribosomal genes, pTA71, would be expected to hybridise predominantly to ribosomal sequences in nDNA, it was considered that it should be possible to determine the proportion of each sample hybridising to the ctDNA probes, P6 and B2, relative to the amount of

Figure 4.3.5.3 Replicate dot blots of total leaf DNA and total endosperm DNA from A grain endosperms (Batch 4):
(i) probed with ctDNA
(ii) probed with P6

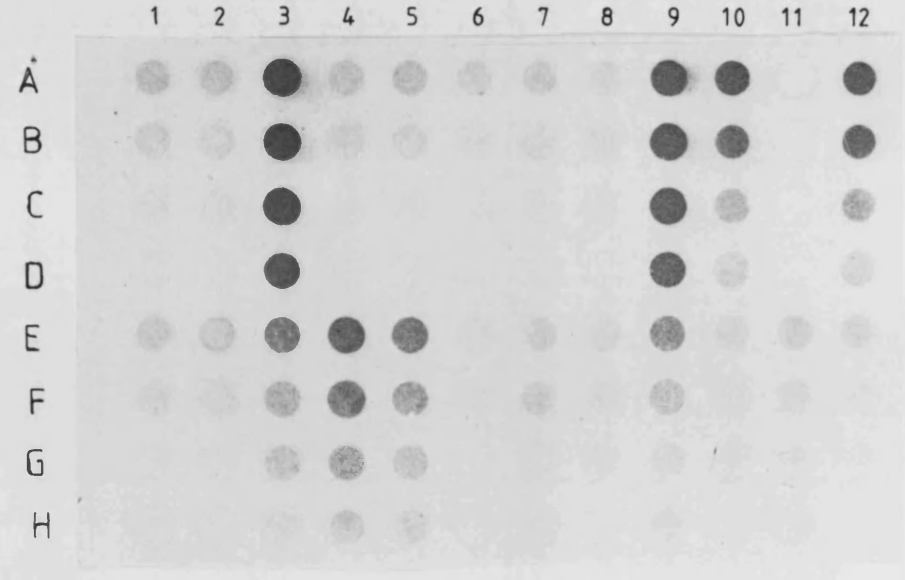
Key

Probe DNA:	(i) 0.12 to 0.001 μg ct DNA	3(A-H), 9(A-H)
	(ii) 4.0 to 0.047 ng P6	
Total leaf DNA (at $4\frac{1}{2}$ wks):		
Sample 1:	0.4 to 0.05 μg	10(A-D), 4(E-H)
Sample 2:	0.4 to 0.05 μg	5(E-H)
Total endosperm DNA at:		
$7\frac{1}{2}$-8 d.p.a.	0.2 to 0.025 μg	1(A-D), 7(E-H)
$8\frac{1}{2}$-9 "	0.4 to 0.05 μg	2(A-D), 8(E-H)
$9\frac{1}{2}$-10 "	"	4(A-D), 11(E-H)
12 "	"	5(A-D), 11(E-H)
14 "	"	6(A-D), 12(E-H)
17 "	"	7(A-D), 1(E-H)
21 "	"	8(A-D), 2(E-H)
Herring sperm DNA:	0.4 to 0.05 μg	11(A-D)
P6 or ctDNA:	(i) 4.0 to 0.047 ng P6	12(A-D)→6(E-H)
	(ii) 0.2 to 0.001 μg ctDNA	

(i)



(ii)



hybridisation of the same samples to the nuclear probe. Results calculated using standard curves for hybridisation to probe DNA (Tables 4.3.5.2 and 4.3.5.3, column 1) were therefore adjusted using a correction factor determined from the amount of hybridisation to pTA71 (Tables 4.3.5.2 and 4.3.5.3, column 2). It was also intended that by comparison of these results it should be possible to determine whether total DNA samples from starchy tissue were hindered from hybridising when filter bound, as was observed for reassociations in solution (section 4.3.3).

Mean estimates of the percentage ptDNA content of endosperms fitted a narrower distribution when calculated relative to the hybridisation of pTA71 than when calculated based on the amount spotted. DNA concentrations were determined by diphenylamine assay but, possibly, the assay results were not as accurate as expected, therefore results related to the nuclear probe may be the more accurate of the two. Despite this, subsequent dot blots were not probed with pTA71 because it was considered that this comparison, although it may reduce variation, may introduce error if the number of genome copies homologous to pTA71 is not constant for all plant organs and developmental stages (Heidecker and Messing, 1986). Possibly selective amplification of the nuclear ribosomal genes may have caused the estimated percentage ptDNA content of leaves to be unusually low. All DNA samples used after this were therefore assayed several times.

Table 4.3.5.2 Results of slot blots of total endosperm DNA from C grains

Source of filter bound DNA	Probe	Estimated % ptDNA Column 1 Absolute values		$\bar{x}$ (σ_{n-1})	Column 2 Values relative to nuclear probe		$\bar{x}$ (σ_{n-1})
Leaf	P6	14.3	13.2	10.95	5.15	4.36	3.78
	B2	7.00	9.3	(4.0)	2.52	3.07	(1.19)
C endosperms at 13 d.p.a.	P6	1.25	0.99	0.89	1.56	1.27	1.11
	B2	0.55	0.76	(0.30)	0.69	0.91	(0.38)
C endosperms at 36 d.p.a. (intact spikes)	P6	2.20	1.86	1.74	2.07	1.22	1.41
	B2	1.61	1.30	(0.38)	1.51	0.85	(0.51)
C endosperms at 36 d.p.a. (degrained spikes)	P6	2.00	1.91	1.71	1.94	1.28	1.40
	B2	1.46	1.45	(0.29)	1.42	0.97	(0.41)
C endosperms at 60 d.p.a. (intact spikes)	P6	0.54	0.36	0.46	0.85	0.76	0.83
	B2	0.45	0.49	(0.08)	0.70	1.03	(0.14)
C endosperms at 60 d.p.a. (degrained spikes)	P6	0.68	0.58	0.61	0.71	0.79	0.73
	B2	0.53	0.65	(0.07)	0.55	0.88	(0.14)
C endosperms at 27 d.p.a.	P6	1.27	0.91	0.80	0.69	1.49	0.84
	B2	0.44	0.56	(0.38)	0.29	0.91	(0.50)
C endosperms at 45 d.p.a.	P6	0.65	0.43	0.42	0.56	0.71	0.50
	B2	0.34	0.27	(0.17)	0.29	0.45	(0.18)

Each estimate is the mean for one filter, of four dots. Background levels of hybridisation of probes to nDNA have not been subtracted (section 4.3.5).

Table 4.3.5.3 Results of slot blots of total endosperm DNA from greenhouse grown A grains

Source of filter bound DNA	Probe	Estimated % ptDNA Column 1 Absolute values		$\bar{x}$ (σ_{n-1})	Column 2 Values relative to nuclear probe		$\bar{x}$ (σ_{n-1})
A endosperms at 9 d.p.a	P6	-			1.18	1.41	0.93
	B2	-			0.48	0.66	(0.44)
A endosperms at 13 d.p.a.	P6	1.49	1.24	1.02	1.10	1.38	0.94
	B2	0.61	0.73	(0.42)	0.45	0.81	(0.40)
A endosperms at 17 d.p.a.	P6	1.07	0.72	0.73	2.16	1.89	1.66
	B2	0.64	0.49	(0.25)	1.29	1.28	(0.44)
A endosperms at 21 d.p.a.	P6	1.89	1.16	1.28	2.46	0.70	1.32
	B2	1.23	0.84	(0.44)	1.60	0.51	(0.90)
A endosperms at 26 d.p.a.	P6	0.20	0.09	0.14	0.40	0.23	0.31
	B2	0.14	0.13	(0.04)	0.29	0.33	(0.07)

Each estimate is the mean for one filter, of four dots. Background levels of hybridisation of probes to nDNA have not been subtracted (section 4.3.5).

Starch contamination of DNA samples

It was not conclusive that starch was inhibiting hybridisation of filter bound DNA from the more mature endosperm tissue. A number of these DNA samples, but not all, did appear to be affected, the rate possibly being reduced by as much as 50% (C grain endosperms from intact spikes at 60 d.p.a. and A grain endosperms at 26 d.p.a.) (Tables 4.3.5.2 and 4.3.5.3). However, DNA extracted from the largest grains, C grain endosperms from degrained spikes at 60 d.p.a., was not significantly affected. On centrifuging solutions of endosperm DNA at 13000 x g for 8 min it was found that some samples yielded an insoluble pellet. An iodine solution stained the pellet blue, indicating the presence of starch. For this reason subsequent DNA extractions were centrifuged as described before assaying the DNA concentration of the supernatant.

Estimated percentage ptDNA of total endosperm DNA

(i) Comparison of endosperms from intact and degrained spikes

Results from endosperms at 13, 36 and 60 d.p.a. indicated that the percentage ptDNA rose from 1.1%, in immature endosperms where cell division was very rapid, to 1.4%, in endosperms where starch deposition was still taking place (Table 4.3.5.2). After this the percentage appeared to fall, possibly to as little as 0.7-0.8% in mature endosperms where the cells had become quite dehydrated. At both 36 and 60 d.p.a., there was no significant difference between

results for intact and degrained spikes: at 36 d.p.a. both samples contained 1.4% ptDNA and at 60 d.p.a. the percentages differed by 12.1±17.7, the endosperms from degrained spikes containing the lower percentage (standard deviations were based on replicate dot blots of the same DNA samples). Further data is necessary to confirm that there is no significant difference between the two spikes.

(ii) Developing A grain endosperms

A grain endosperms showed a similar trend to C grains, the percentage ptDNA increasing from 0.93% at 9 and 13 d.p.a. to 1.66% at 17 d.p.a., when starch synthesis was rapid, and then falling again, as the endosperms dehydrated, to as little as 0.31% (Table 4.3.5.3).

It was not possible to deduct background levels of hybridisation to nDNA from these figures since no data was available for filters washed at this stringency (section 4.3.6).

4.3.6 Further dot blots

The following dot blots were carried out in order to obtain a more accurate estimate of the changes in percentage ptDNA as endosperms develop and also to determine the background levels of hybridisation of both P6 and B2 to nDNA.

Unlike the previous dot blots which were probed with [^{32}P]DNA, these filters were probed with [^{35}S]DNA. Initially a number of filters and DNA samples were wasted due to the

higher levels of non-specific background hybridisation of the ^{35}S (see Fig., 4.3.5.3(ii)). Several steps were taken to reduce the problem: (i) additional sonicated salmon testes DNA was added to the Hybridisation buffer to 500 μg/ml; (ii) radiolabelled probes were ethanol precipitated after purification from [^{35}S]dCTPαS on Sephadex G50 columns and (iii) the wash stringency was increased, filters being washed in 0.1 x SSC at 50°C rather than just 3 x SSC at 60°C (sections 4.2.8 and 4.2.10). Filter backgrounds were reduced largely by changing the wash stringency.

Percentage ptDNA in wheat leaves

The percentage ptDNA contents of two total leaf DNA preparations from $4\frac{1}{2}$ week old leaf tissue did not appear to be significantly different, at 11.2% and 11.7%, respectively (Table 4.3.6.1). The estimated percentage ptDNA varied depending on whether the filters were probed with P6 and B2 or the whole plasmids, pTacP6 and pTacB2 (both pBR322 derivatives). The latter gave threefold greater estimates when the standard curves were also whole plasmid: it appeared that the pTac plasmids hybridised proportionately more to the total DNA samples than they did to themselves. This could be because the standard curve plasmid DNA concentration may have been in excess over that in solution therefore hybridisation was limited by the rate of probe diffusion, rather than the nucleation event (Anderson and Young, 1985) (see section 4.3.5). An alternative explanation, which is more likely to account for such a large increase, is that the single

Table 4.3.6.1 Estimated percentage ptDNA in wheat leaf DNA samples

Leaf DNA	Probe	Standard	Estimated % ptDNA	Means (standard deviation)	
Sample 1	P6	P6	*14.3		
	"	"	*13.2	11.2	
	"	pTacP6	12.0	(2.98)	
	B2	pTacB2	* 7.0		
	"	"	* 9.3		
Sample 2	P6	pTacP6	19.1		11.46
	"	"	5.2		( )
	"	"	8.3		
	"	"	7.7	11.7	
	"	"	11.8	(4.5)	
	"	"	13.5		
	B2	pTacB2	14.1		
	"	"	13.9		
Sample 1	pTacB2	pTacB2	23.0		
Sample 2	pTacP6	pTacP6	41.8		
	pTacB2	pTacB2	23.0	36.0	
	"	"	39.1	(8.7)	
	"	"	39.9		

*data from slot blots (section 4.3.5)

Estimates are means of 8 or 16 dots on individual filters. Background levels of probe hybridisation to nDNA have not been subtracted.

stranded plasmid DNA tails of the probe, renatured to filter bound ptDNA as opposed to whole plasmid DNA, would be free to reassociate with a larger proportion of the probe; thereby resulting in an amplified signal (Anderson and Young, 1985).

Because of this variation, and because despite these problems the relative amounts of hybridisation to the total endosperm and total leaf DNA samples remained constant, the figure of 11.46% ptDNA in these leaf samples was used as a standard for estimating the percentage ptDNA in endosperm tissue. This was also possible since there was no significant difference between the estimates of percentage ptDNA in leaves for filters probed with either P6 or B2.

Hybridisation of P6 and B2 to wheat nuclear DNA

Two samples of nDNA were prepared from wheat embryos and probed with P6, alongside total leaf DNA samples and an herring sperm DNA control. The results indicated that the nDNA preparations contained up to 1.5% of sequences with homology to ctDNA (Table 4.3.6.2).

Table 4.3.6.2 Results of dot blots of nDNA probed with P6

Source of filter bound DNA	Estimated % ptDNA
NDNA (sample 1)	1.50
NDNA (sample 2)	1.45
Herring sperm DNA	not detectable

Results are means of eight dots.

It was considered unlikely that all of this hybridisation could be to nDNA sequences alone since estimates of the ptDNA content of endosperm samples had given figures of less than 1.5% (section 4.3.5). The Southern blots had clearly indicated the presence of ptDNA in wheat endosperm, in significant amounts (section 4.3.2). It was therefore concluded that the nDNA preparations must have been contaminated with ptDNA from embryo proplastids or amyloplasts (Lamppa and Bendich, 1979a): the extraction procedure adopted may have enriched for plastid contamination.

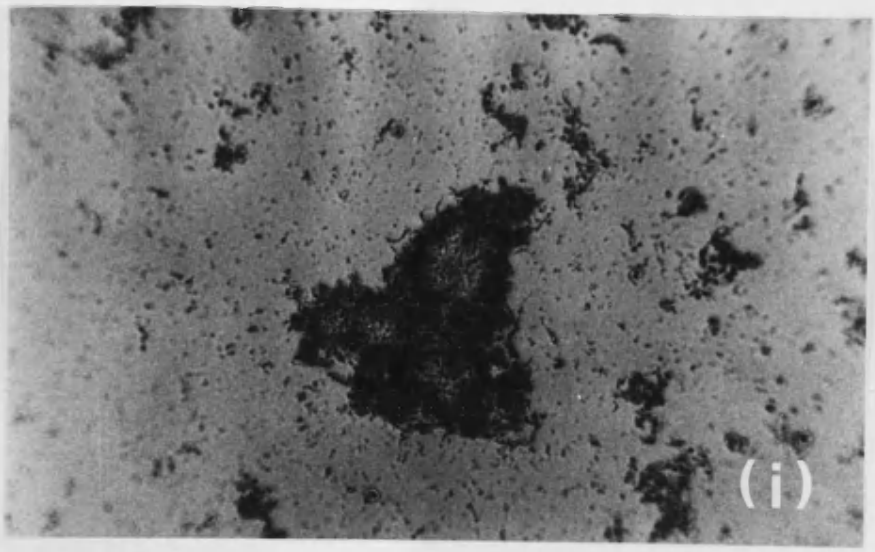
The nuclei, when prepared, were stained with DAPI, ethidium bromide or iodine to look for plastids and starch granules (Fig. 4.3.6.1). It was found that the nuclei were not intact as expected (Luthe and Quatrano, 1980) but had clumped together (Fig. 4.3.6.1(i)). Since the preparations appeared so damaged, the fluorescence, in DAPI stained preparations, may have been associated with both chromosomal and cytoplasmic debris (Fig. 4.3.6.1(ii)). Few large starch granules or plastids were observed but iodine staining revealed many purple stained spherical objects of 2-4 μm which may have been small starch granules or proplastids containing starch (Sandstedt, 1946). (These can be seen only poorly in Fig. 4.3.6.1(iii), surrounding the dark nuclei).

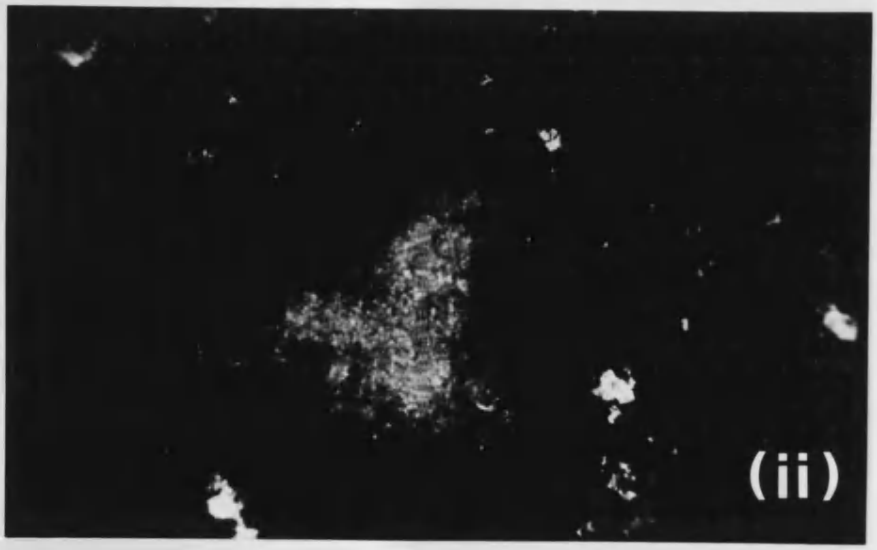
Rather than attempt to prepare nuclei free from plastid contamination, it was decided that the nDNA should be restricted with an enzyme which specifically cleaves non-

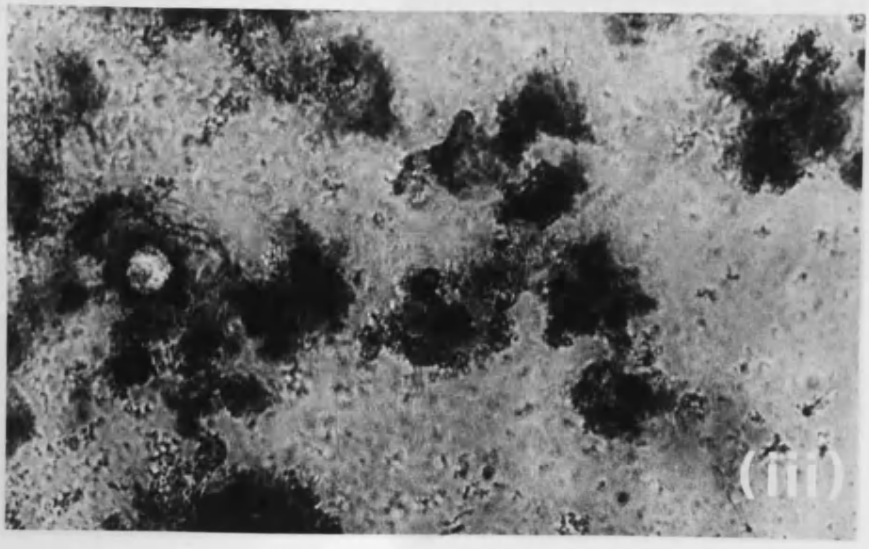
Figure 4.3.6.1 Nuclei prepared from wheat embryos: (i) Clumps of nuclear material and cell debris under tungsten light, (ii) the same material, DAPI stained, under fluorescent light, and (iii) nuclei stained with iodine to reveal starch contamination.

(magnification: (i) and (ii) 155 μm)

((iii) 39 μm)


(i)


(ii)


(iii)

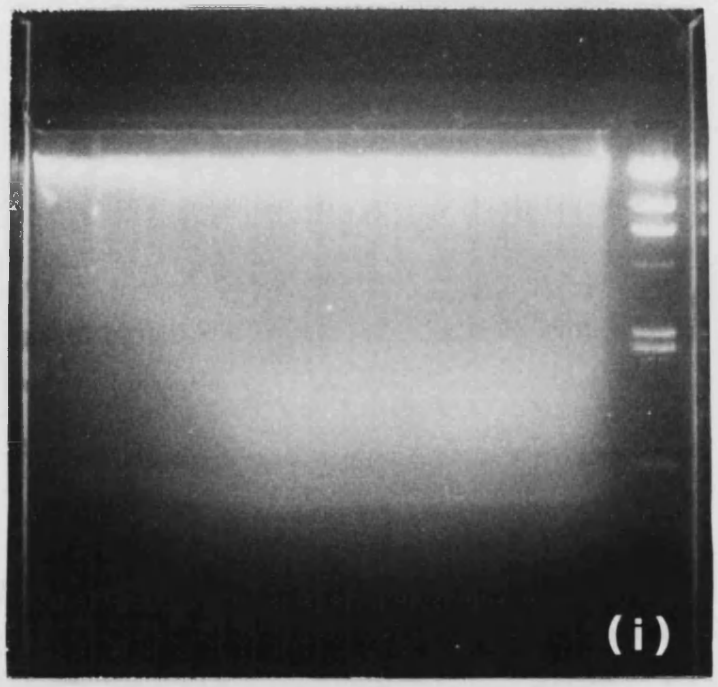
methylated sequences, such as found in ptDNA, leaving heavily methylated nDNA reasonably intact (Kessler and Höltke, 1986). HpaII restricted nDNA (sample 2) was electrophoresed (Fig. 4.3.6.2(i)) and only DNA of 11-20 kb was recovered (Fig. 4.3.6.2(ii)). The DNA had been extensively digested making it unlikely that the nDNA would still be contaminated with ptDNA.

Duplicate dot blots of the restricted, recovered nDNA were probed with both pTacP6 and pTacB2 (Table 4.3.6.3). For the pTacP6 probed filter the percentage ptDNA was determined by comparison with two standards: total leaf DNA (11.46%) and total endosperm DNA from A grains at 21 d.p.a. (1.12%). For the pTacB2 probed filter only the endosperm standard was used: hybridisation was poor so results were determined by plotting peak height of densitometer scans versus DNA loaded and then comparing the gradients.

Controls had shown that at the stringency used here there was no detectable cross homology between pBR322 or herring sperm DNA (Fig. 4.3.5.3) and total wheat endosperm or nuclear DNA (Fig. 5.3.3.2(ii) and section 5.3.3, Southern blot not presented). It is therefore apparent that the pBR322 sequences of the pTac plasmids P6 and B2 contain little, if any, homology to the nuclear genome of wheat.

It is not clear why the non-restricted nDNA probed with pTacP6 failed to hybridise as strongly as before but the standards both suggest that the restricted nDNA contained up to 0.33 to 0.43% of sequences with homology to ctDNA, considerably less than the previous estimates. Similarly the

Figure 4.3.6.2 Preparation of HpaII nDNA: (i) Digested nDNA electrophoresed through an 0.5% agarose gel and (ii) Recovery of high molecular weight (16-20 kb) nDNA. Marker in final and middle tracks, respectively, is HindIII lambda DNA.


(i)

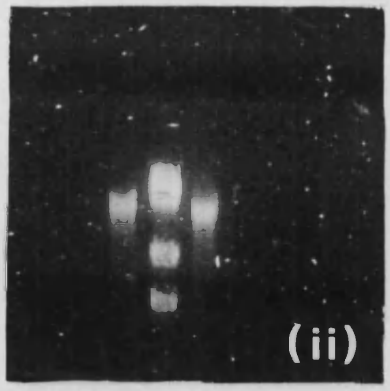

(ii)

Table 4.3.6.3 Results of dot blots of HpaII restricted DNA

Source of filter bound DNA	Probe	Standards used	Estimated % ptDNA	
Not restricted	pTacP6	Total leaf	0.980	
nDNA (sample 2)		Total endosperm	0.755	
	pTacB2	Total endosperm	1.65	
HpaII restricted	pTacP6	Total leaf	0.429	0.38
nDNA (sample 2)		Total endosperm	0.330	
	pTacB2	Total endosperm	0.34	

Results are means of eight dots on each filter

filter probed with pTacB2 suggested 0.34% homologous sequences.

Comparison of endosperms from intact and degrained spikes

Results from two dot blots were quite variable (Table 4.3.6.4): in addition those probed with P6 gave higher estimates of the percentage ptDNA than had been previously determined (Table 4.3.6.1). Subsequent results, from A grain endosperms, confirmed that the earlier, lower estimates were probably more representative. Due to the discrepancy between the data from the two probes it was not possible to detect a significant difference between endosperms from intact and degrained spikes. This discrepancy was not found in other P6 and B2 comparisons.

From this data and the figures presented in section 4.3.5 (Table 4.3.5.2), it was not evident that there was any difference in the percentage ptDNA of endosperms from intact and degrained spikes which was maintained throughout development. However a small difference of 10%, for example, would be difficult to detect without replicate DNA samples at different developmental stages and a number of dot blots and, unfortunately, there was insufficient plant material remaining for further dot blots of DNA from endosperms from intact and degrained spikes.

Percentage ptDNA in A grain endosperms

Estimates of the percentage ptDNA of A grain endosperms, from two batches of plants, were derived from nine dot blots

Table 4.3.6.4 Results of dot blots of total endosperm DNA from C grains

Source of filter bound DNA	Probe	Estimated % ptDNA	% ptDNA less background hybridisation to nDNA	$\bar{x}$
Endosperms at 13 d.p.a.	P6	2.50	2.12	1.60
	pTacB2	1.41	1.07	
25 d.p.a. from intact spikes	P6	2.61	2.23	1.39
	pTacB2	0.88	0.54	
25 d.p.a. from degrained spikes	P6	1.93	1.55	1.09
	pTacB2	0.96	0.62	
45 d.p.a. from intact spikes	P6	1.43	1.05	0.67
	pTacB2	0.62	0.28	
45 d.p.a. from degrained spikes	P6	1.96	1.58	1.14
	pTacB2	1.03	0.69	

Estimates are means of eight dots on individual filters.

Table 4.3.6.5 Results of dot blots of total endosperm DNA from greenhouse grown A grains

Source of filter bound DNA	Probe	Estimated % ptDNA		$\bar{x}$ less background hybridisation of probe to nDNA (σ_{n-1})
Endosperms at:				
5 d.p.a.	P6	0.52	0.81	0.33(0.20)
	B2/pTacB2	0.89	0.53	
7 d.p.a.	P6	1.01	1.70	1.06(0.33)
	B2/pTacB2	1.30	1.67	
17 d.p.a.	P6	0.99	1.46	0.92(0.24)
	B2/pTacB2	1.47	1.20	
21 d.p.a.	P6	1.01	1.43	0.85(0.18)
	B2/pTacB2	1.27	1.14	
23 d.p.a.	P6	0.92	1.47	0.88(0.25)
	B2/pTacB2	1.20	1.38	
26 d.p.a.	P6	0.98	1.26	0.90(0.22)
	B2/pTacB2	1.40	1.41	
29 d.p.a.	P6	0.78	1.12	0.57(0.14)
	B2/pTacB2	0.94	0.88	

Estimates are means of eight dots as individual filters.

Table 4.3.6.6 Results of dot blots of total endosperm DNA from growth cabinet grown A grains

Source of filter bound DNA	Probe	Estimated % ptDNA			$\bar{x}$ less background hybridisation of probe to nDNA (σ_{n-1})
Endosperms at:					
$7\frac{1}{2}$-8 d.p.a.	P6	1.79	1.04	1.15	0.83(0.35)
	B2/pTacB2	1.14	-	0.82	
$8\frac{1}{2}$-9 d.p.a.	P6	1.52	0.95	1.42	0.86(0.26)
	B2/pTacB2	1.29	-	0.92	
$9\frac{1}{2}$-10 d.p.a.	P6	1.33	0.96	1.19	0.68(0.23)
	B2/pTacB2	0.77	-	0.97	
12 d.p.a.	P6	1.12	1.05	1.36	0.78(0.12)
	B2/pTacB2	1.10	-	1.07	
14 d.p.a.	P6	1.26	0.83	1.12	0.69(0.17)
	B2/pTacB2	0.98	-	0.98	
17 d.p.a.	P6	1.25	1.04	1.60	0.87(0.21)
	B2/pTacB2	1.09	-	1.18	
21 d.p.a.	P6	1.80	1.13	1.83	1.02(0.45)
	B2/pTacB2	0.75	-	1.41	

Estimates are means of eight dots on individual filters.

probed with either P6 or B2. Results are presented in Tables 4.3.6.5 and 4.3.6.6 and mean percentages are plotted against grain fresh weight (Fig. 4.3.6.3).

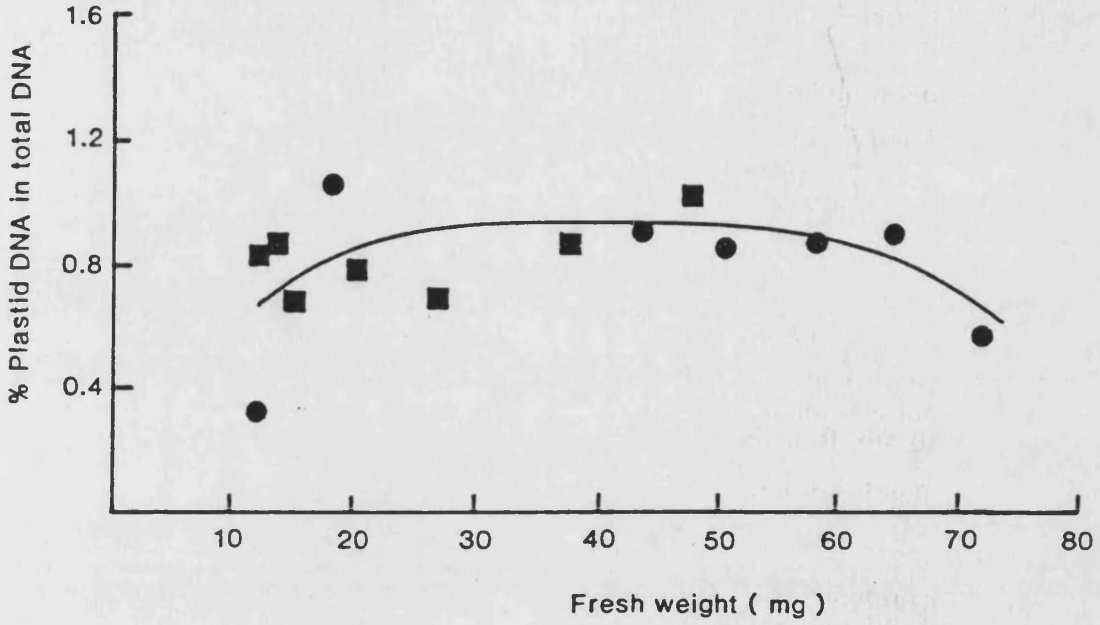


Figure 4.3.6.3 Percentage plastid DNA content of A grain endosperms throughout grain filling:
(●) greenhouse grown grains
(■) growth cabinet grown grains.

Throughout the periods of rapid cell division and starch synthesis for grains weighing from 18 to 72 mg there was no more than a two fold variation in endosperm percentage ptDNA. Furthermore, for grains weighing from 35 to 65 mg the maximum and minimum percentages differed by only 0.17%, from 0.85% to 1.02%. The mean ptDNA content of 0.91% for these endosperms may even extend to those from grains weighing as little as 18 mg or less.

It is not possible to be certain of the mean fresh weight of grains at the younger ages, since for grains in which the endosperms were extremely small, their contribution to the total extract was negligible. Therefore the estimated 0.33% ptDNA for endosperms from grains weighing 12.5 mg (5 d.p.a., greenhouse grown) may be correct, despite higher estimates for grains of a similar or slightly larger size. (For the other sample from grains weighing 12.5 mg ($7\frac{1}{2}$-8 d.p.a., growth cabinet grown) the standard deviation was high due to one very high estimate.) It was concluded that there may be an increase in the percentage ptDNA content of young endosperms during the period when proplastids are rapidly differentiating into amyloplasts.

Since none of the grains used for these extractions were fully mature (not even those weighing 72.5 mg, since the grains were still green), it is possible that as the endosperms dehydrated the percentage ptDNA may have fallen. This was suggested by earlier dot blot results using more mature endosperms (this section and section 4.3.5) and by the estimated 0.57% ptDNA content of endosperms weighing 72.5 mg.

However, it is possible that these results indicate a decline simply because the ptDNA may become more closely associated with the starch granules as the tissue dehydrates, thereby making the ptDNA more difficult to extract. Previous results had suggested that the percentage ptDNA may rise in developing endosperms, reach a maximum and then decline as the endosperm dehydrates (section 4.3.5). The data presented here also indicate that the percentage may initially rise, plateau and then fall in the most mature endosperms; but throughout most of grain filling and endosperm development the percentage remains quite constant at 0.90% (Fig. 4.3.6.3).

4.3.7 Plastome copy number throughout endosperm development

From the graph of the percentage ptDNA in total endosperm DNA versus grain fresh weight (Fig. 4.3.6.3) it was possible to estimate the percentage ptDNA for endosperms at different developmental stages. These average figures were used to estimate the total endosperm ptDNA content (Fig. 4.3.7.1), number of plastome copies per cell (Fig. 4.3.7.2) and the number of plastomes per A type amyloplast and per starch granule (Fig. 4.3.7.4). In order to make these calculations it was necessary to assume that background hybridisation of the probes, P6 and B2, to mtDNA did not constitute a significant proportion of the percentage ptDNA detected since only background hybridisation to nDNA had been deducted. This assumption is discussed in section 4.4.4 and it is suggested that the possible percentage that may be

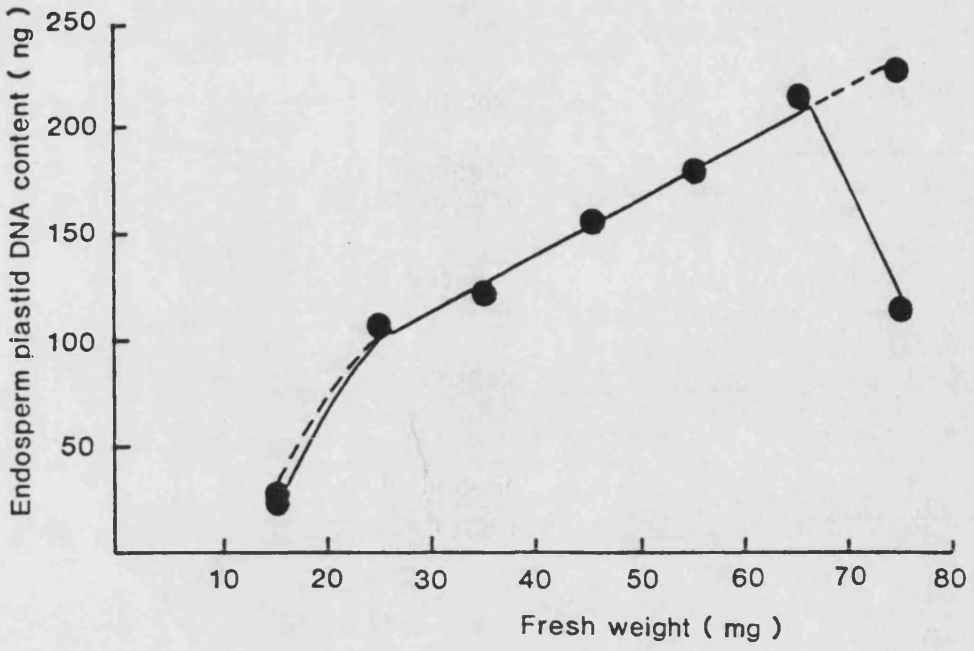


Figure 4.3.7.1 Endosperm plastid DNA content during grain development. Two points are presented for very young and mature endosperms due to the uncertainty as regards the percentage ptDNA at these stages.

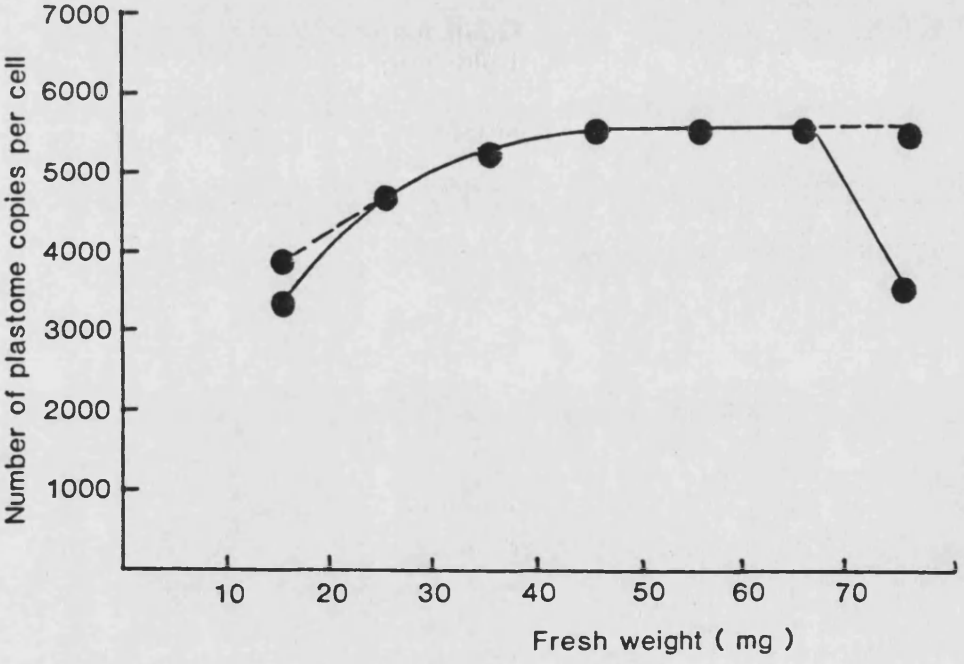


Figure 4.3.7.2 Number of plastome copies per cell during endosperm development. See legend for Fig. 4.3.7.1.

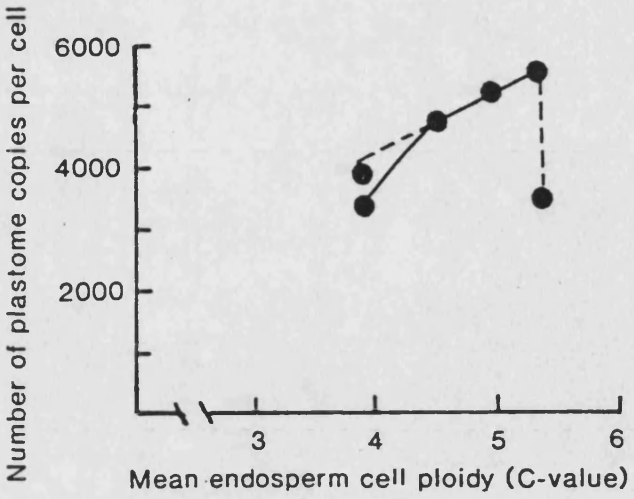


Figure 4.3.7.3 Cell plastome number plotted versus mean cell ploidy. See legend for Fig. 4.3.7.1.

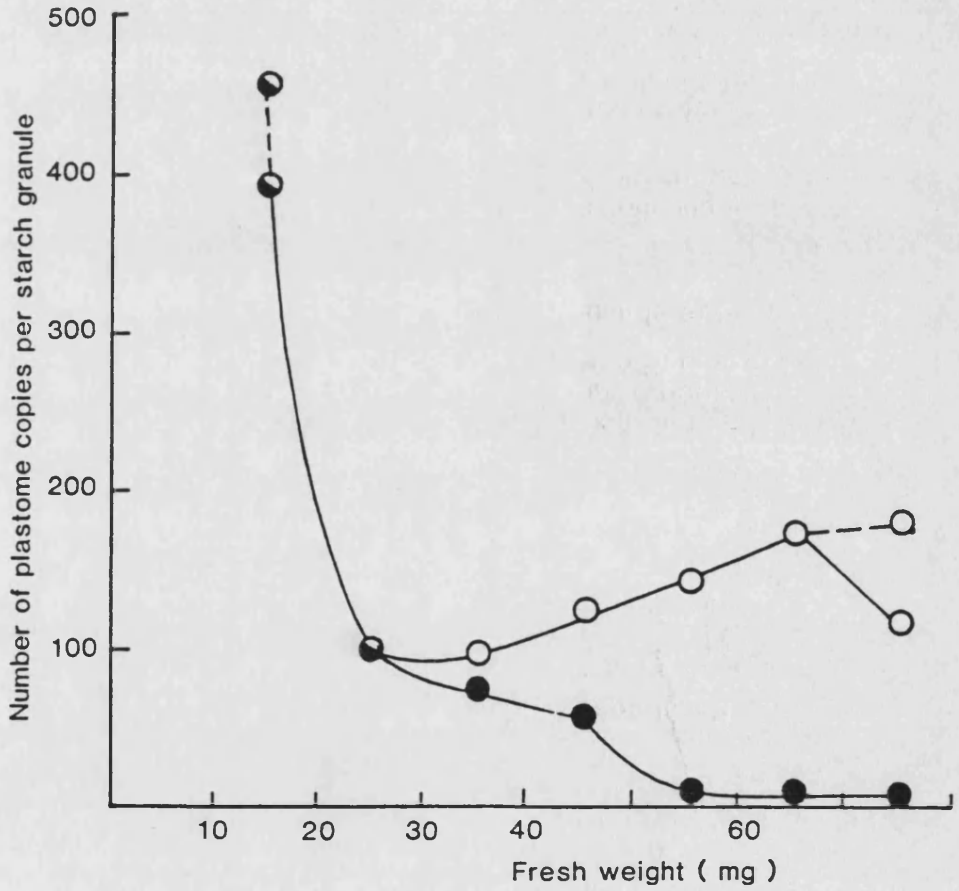


Figure 4.3.7.4 Number of plastome copies per starch granule (●) and per A type amyloplast (○).
See legend for Fig. 4.3.7.1.

attributed to probe cross-hybridisation, rather than the whole plastid genome, may be between 5.8 to 8.7% of the estimated 0.91% ptDNA.

The total endosperm complement of ptDNA increased throughout grain development, at least until grain fresh weight reached 60-70 mg, the rate appearing to be almost linear for grains of 20-30 mg upwards with a more rapid increase occurring in younger grains of 10-20 to 20-30 mg (Fig. 4.3.7.1). The increase in endosperm ptDNA was similar to those for total endosperm DNA content (Fig. 2.3.3.2) and cell number (Fig. 2.3.6.2) which showed an almost linear increase from 10-20 mg to 60-70 mg.

The number of plastome copies per cell (Fig. 4.3.7.2) appeared to parallel the trend for mean cell ploidy (Fig. 2.3.4.2), since a plateau of around 5560 plastomes per cell was reached in grains of 40-50 mg which was maintained until 60-70 mg, if not later in development. This is more clearly demonstrated by Fig. 4.3.7.3 which reveals that an increase in mean ploidy was accompanied by an increase in the cell plastome content and that when the former ceased so did the latter. Due to the uncertainty as regards the percentage ptDNA content of grains of just 10-20 mg it is not possible to discern whether the curve is linear from this stage. However, if a linear relationship exists over this period it appears that a doubling in mean ploidy is accompanied by an increase in the plastome content of the same order. It is also not clear whether this relationship may cease in the maturing endosperm where it is possible that the ptDNA

content of the endosperm may be selectively degraded (Fig. 4.3.6.3).

Plastome number is clearly not tightly linked to the total number of starch granules (Fig. 4.3.7.4) since when the B type granules were initiated in grains of 40-50 to 50-60 mg (Fig. 3.3.1.2) there is no corresponding increase in ptDNA, consequently the ratio of plastomes to granules falls from around 70 to less than seven. However, there does appear to be a closer relationship between the A type granules and the plastome complement. For grains weighing 30-40 to 60-70 mg the number of plastome copies per A type increases linearly with increasing grain weight (Fig. 4.3.7.4), coincident with the rapid increase in A type modal volume (Fig. 3.3.1.10). Since A type amyloplast number was estimated from the number of starch granules detected in the young endosperm (see sections 3.3.2 and 3.4.1), any plastid not containing a starch granule was not included in the estimates. Thus, although the ratio of plastomes to each A type granule is approximately 400 in grains weighing 10-20 mg, if these plastomes are partitioned between the total plastid number in young endosperms, including proplastids and very immature amyloplasts, the figure is likely to be considerably reduced.

4.4 Discussion

4.4.1 DAPI staining of endosperm amyloplasts

The specificity of DAPI for double stranded DNA, in particular the A-T rich regions (Kapuscinski and Szer, 1979) has been well documented (James and Jope, 1978). As a consequence it has proven very suitable for locating DNA within the cell and different organelles and, since the fluorescence of the bacteriophage T5 has been observed by DAPI-staining (James and Jope, 1978), it is evident that the stain is also sufficiently sensitive to locate as little as one ptDNA molecule, provided the DNA is sufficiently compact. Investigations staining plant tissues, protoplasts and isolated organelles with DAPI have revealed that ptDNA tends to be localised in small, spherical, dumb-bell or rod shaped 'nucleoids' of 0.2 to 1.2 μm (Kuroiwa et al., 1981), probably containing upwards of 2 to 3 molecules of DNA (Hansmann et al., 1985; Steele Scott and Possingham, 1980). These nucleoids also vary in number and in spatial and temporal distribution within the different plastids of the plant kingdom (Kuroiwa et al., 1981).

Wheat chloroplast nucleoids have been DAPI stained and found to number between seven to 16 per chloroplast and to be organised in a narrow, discontinuous band (Sellden and Leech, 1981). These results were confirmed by the light micrographs presented in Fig. 4.3.1.1. Sellden and Leech (1981) also found that in younger plastids the DNA was organised in an almost continuous band around the plastid periphery. More complete, ring-shaped DAPI staining regions have been

visualised, and even isolated (Kuroiwa and Suzuki, 1981), from the chloroplasts of a variety of different algae (Kuroiwa et al., 1981; Ersland et al., 1981; Nagashima et al., 1984).

A similar search for wheat amyloplast nucleoids revealed the probable existence of endosperm amyloplast DNA (Fig. 4.3.1.3), due to the presence of fluorescence around some, but not all, of the larger, A type starch granules. The DNA appeared to be located not so much in discrete nucleoids but as more diffuse fluorescence with several more intensely staining regions on the equatorial groove and, possibly, on the apex of the granule. That this fluorescence represented only ptDNA was not conclusive since DNase treatment of the amyloplast preparation had failed to remove all extraneous DNA (cf. Selldèn and Leech (1981) where a 2.5 fold lower DNase concentration successfully yielded clean, intact chloroplasts).

Macherel et al. (1985) in a study of Acer pseudoplatanus amyloplasts, both in situ and isolated, revealed a large and irregular DAPI staining pattern, which more closely resembled the A type amyloplasts, whereas in the mitochondria the nucleoids were smaller and more compact (Kuroiwa et al., 1981). Steele Scott et al. (1984) compared the nucleoids of potato tissues and found that the large, compact, spherical nucleoids of young tuber cells, containing either small or no starch granules, were no longer evident in the starch filled cells from more mature tubers. The latter, when DAPI-stained, appeared very similar to wheat endosperm

protoplasts (Fig. 4.3.1.3(ii)), containing diffuse fluorescent regions around the periphery of the large starch granules. There are few other descriptions of DAPI-stained, non-photosynthetic plastids: Steele Scott et al. (1982) reported that undifferentiated plastids of an albino barley mutant contain a single, large nucleoid, in contrast to the many peripherally located nucleoids of the normal barley chloroplast. In addition, Hansmann et al. (1985), in a thorough study of Narcissus pseudonarcissus chromoplasts and chloroplasts, determined that the numbers, packaging and protein content of nucleoids appeared to be related to gene expression.

Digitonin treatment (at 0.11% w/v) of wheat endosperm protoplasts did not succeed in increasing the yield of intact amyloplasts (Fig. 4.3.1.3). This treatment was included because digitonin (a sterol glycoside) is a mild detergent which has been found to permeabilise sterol-containing, plasma membranes (Zuurendonk et al., 1979), leaving mitochondria and secretory organelles intact (Perrin et al., 1987). However, it has also been used at 0.5% w/v to dissociate certain proteins from thylakoid membranes and to isolate the chloroplast photosystems from isolated chloroplasts (Kirk and Tilney-Bassett, 1978), therefore it may be that digitonin is not sufficiently specific for the purpose described above. A more successful alternative might be L-α-lysophosphatidyl choline which has been found to permeabilise the cells of Glycine max without affecting plastid DNA synthesis (Cannon et al., 1986). The resultant

cells were permeable to DNase I as well as being capable of RNA and protein synthesis.

Better DAPI staining results may have been obtained had the protoplast and organelle preparations been fixed in glutaraldehyde prior to staining (Hansmann et al., 1985; Kuroiwa et al., 1981).

4.4.2 Further evidence for the existence of amyloplast DNA in wheat endosperm

The existence of nucleoids in amyloplasts in sycamore cell cultures (Macherel et al., 1985), potato tuber (Steele Scott et al., 1984) and the cotyledon and endosperm of castor bean (Galli et al., 1986) has been indicated and that these contain DNA homologous to the ctDNA of the same species has been suggested (Macherel et al., 1985, 1986b; Steele Scott et al., 1984).

Southern blots of PstI restricted total endosperm DNA probed with the ctDNA fragments P6 and P7 revealed single fragments comigrating with those from PstI restricted total leaf and ctDNA. These results helped to confirm the presence of amyloplast DNA in this tissue (Figs. 4.3.2.2 and 4.3.2.3). Analyses of the restriction enyzme digests of the plastome from other organelles (Iwatsuki et al., 1985; Macherel et al., 1985) have indicated that the plastome does not change between different organelles, within a species, but that differential nuclear and plastid gene expression controls plastid transition (Parthier, 1982; Walbot et al., 1980; Sundqvist et al., 1980). In leaves this control may be

effected by light (Richter, 1984; Jenkins et al., 1983; Miller et al., 1983; Cannon et al., 1985a) and a similar transition is seen in potato tubers (Steele Scott et al., 1984; Conover and Pryke, 1987). The control of plastid transition as a ripening response in fruits is also being investigated (Piechulla et al., 1986).

Dot blots of total endosperm DNA probed with P6 and B2 revealed that a proportion of the DNA hybridised strongly to these fragments, which when adjusted to account for the fraction of the plastome that these probes comprise, indicated the presence of up to 1.27% ptDNA in the endosperm. Subsequent correction for the fraction hybridising to the nuclear genome (sections 4.3.6 and 4.4.4) gave estimates of 0.91% ptDNA in wheat endosperm throughout the grain filling period of development (Fig. 4.3.6.3).

4.4.3 Technical problems associated with reassociation reactions, dot blots and ctDNA preparations

Reassociation kinetics using whole ctDNA probes have been successfully used to quantify ptDNA amounts of less than 1% of total DNA extracted from higher plant tissues, including pea roots (Lamppa and Bendich, 1979a) and spinach root nuclei (Timmis and Steele Scott, 1983). However difficulties were encountered in this project as bi- or triphasic Cot curves were obtained, which when plotted as the reciprocal of the single stranded component against the log Cot revealed two or more linear rates. Although this pattern was similar to that described for heat bleached

Euglena mutants (Hussein et al., 1982), which the authors claim were a result of repeated rDNA sequences in these atypical genomes, the same explanation is inadequate here. Since both dot blot probes used in this thesis, P6 and B2, hybridised similarly to both total leaf and endosperm DNAs and since the existence of homology between a fraction of wheat endosperm DNA and the majority of chloroplast plastome fragments has been shown (Fig. 5.3.3.2(ii)), it is unlikely that sections of the amyloplast genome were preferentially amplified to the extent indicated. Rather, it is more probable that the ctDNA probes used were insufficiently pure: restriction enzyme digests of these were poor (not presented) and hybridisation to both nDNA and ctDNA cloned fragments had indicated contamination (section 4.3.5).

An alternative extraction technique for wheat ctDNA is the non-aqueous procedure described by Bowman and Dyer (1982) which claims a tenfold yield improvement. However, this method was demonstrated to yield ctDNA of 75% purity which is inadequate for reassociation reactions. It was suggested that purity may be increased by isopycnic CsCl and ethidium bromide density gradient centrifugation and a number of authors propose that this may be more effective if carried out twice (Kolodner and Tewari, 1975; Vedel et al., 1976). Despite this, Vedel and Quetier (1978) found the restriction pattern of the lower and upper bands were the same and results presented here indicated that the lower band, taken after a single centrifugation, was not pure ccctDNA (section 4.3.5). It has also been found that improved yields of intact

chloroplasts, and therefore ctDNA, can be obtained from cereal protoplasts (Karimov et al., 1978; Leegood and Walker, 1983) and it has been suggested that EDTA should be omitted as it may promote the activity of endogenous wheat DNases (Jones and Boffey, 1984).

However, it was not only purity but also good yields that were necessary, since sufficient probe DNA is essential for adequate concentration determinations and for standard ctDNA reassociations. It was for this reason, and also due to potential problems whereby endosperm starch may affect reassociation rates in solution (section 4.3.3) that it was decided that dot blots using cloned ctDNA fragment probes would facilitate the determination of the percentage ptDNA in wheat endosperm. Similar procedures have now been used in other laboratories (Steele Scott et al., 1984; Aguettaz et al., 1987; Bowman, 1986). The principal advantages of the dot blot technique are that replication is easy, requiring considerably less DNA per sample, larger numbers of samples can be processed together and assay is more rapid. It was found that hybridisaton of the radioactive probe was most easily and accurately quantified by counting filter squares in liquid scintillant (Cannon et al., 1985b), rather than by densitometric scans of autoradiographs (section 4.3.5). The main disadvantage of dot blots is that lower levels of probe contamination, with sequences abundant in the filter bound DNA, are likely to give amplified signals, thereby increasing error, therefore the use of cloned ctDNA probes was imperative (Fig. 4.3.5.3 cf. (i) and (ii)).

4.4.4 Sequence homologies between the plastid, nuclear and mitochondrial genomes

For the levels of hybridisations of cloned fragment probes to be representative of the number of plastid genome copies it is necessary that they should hybridise only to the plastid genome or, if not, that the fraction hybridising to other genomes is quantified and subtracted. The main advantage of using the ctDNA fragment P6 as a probe was that since it comprises a part of the inverted repeat of the plastome (Bowman *et al.*, 1981) it would hybridise twice as strongly, compared with fragments from the single copy regions, thereby facilitating the accurate detection of low levels of ptDNA. However, since it encodes the ribosomal genes it was considered possible that cross hybridisation to rDNA from other cell compartments may be a problem (Pring and Lonsdale, 1985), therefore the B2 fragment, spanning the *rbcL* gene was selected for parallel experiments. In order to minimise the contribution of sequences with low homology, dot blot filters were washed vigorously under fairly stringent conditions (0.1 x SSC, 0.1% SDS, 50°C).

Over the past five years results have revealed that both the nuclear and mitochondrial genomes contain regions which are considered to have originated from the plastome (Pring and Lonsdale, 1985). In addition, cross homology between the nuclear and mitochondrial genomes has been detected in a number of organisms (Gellisen *et al.*, 1983; van den Boogaart *et al.*, 1982).

Stern and Lonsdale (1982) were the first to report a 12 kbp sequence in maize mtDNA which contained a region of 90% homology to the 16S rRNA region of the chloroplast genome. Subsequently, regions of homology to the rbcL gene (Lonsdale et al., 1983) and to the chloroplast P700 chlorophyll a apoprotein (Whisson and Steele Scott, 1985) were also detected in the maize mitochondrial genome. As regards the nuclear genome it was found that a 3.0 Kbp XhoI fragment containing part of the 23S rRNA gene of spinach showed homology to spinach nDNA (Timmis and Steele Scott, 1983), as did an 1.75 kbp EcoRI fragment covering the rbcL gene, the former to five and the latter very weakly to four bands in nDNA EcoRI digests (Steele Scott and Timmis, 1984). More recently, evidence has been presented which suggests that the nuclear genome may contain homologous sequences to the majority of the plastome (Stern and Palmer, 1983), and that the nuclear genome is heavily methylated in these regions (Steele Scott and Timmis, 1984; Timmis and Steele Scott, 1983).

The nuclear genome

The levels of cross hybridisation of the fragments P6 and B2 to the wheat nuclear genome were quantified by dot blot hybridisation (Table 4.3.6.3). They were found to hybridise to a level equivalent to 0.38% and 0.34% ptDNA, respectively, despite the absence of any hybridisation of either to herring sperm DNA. These figures were determined using DNA extracted from embryo nuclei which had been HpaII

restricted to remove contaminating plastomes. HpaII, which selectively restricts unmethylated DNA (Kessler and Höltke, 1986; Gruenbaum et al., 1981a,b), has been used previously to restrict ptDNA and mtDNA to fragments of less than 4.0 and ≥3.9 kbp, respectively, leaving nDNA largely intact (Timmis and Steele Scott, 1983; Macherel et al., 1985). This is because 90% of the HpaII recognition sequences have been found to be methylated at the internal cytosine (CCGG) in wheatgerm DNA (Gruenbaum et al., 1981b). In sycamore amyloplast DNA neither of the cytosine residues appeared to be methylated, as evidenced by the identical MspI and HpaII restriction digests (Macherel et al., 1986; Gruenbaum et al., 1981b).

An alternative nDNA extraction procedure, which avoids the use of Triton X100, on the basis that it permeabilises the nuclear membrane (Guilfoyle et al., 1986), may be more suitable for the isolation of unclumped, debris free nuclei (cf. Fig. 4.3.6.1) (see also Dunham and Bryant, 1983).

For spinach, reassociation kinetics, using [^{32}P]ptDNA and nDNA from roots, were used to estimate the number of plastome equivalents per haploid nuclear genome. Results indicated five or six copies, with the percentage of the total comprising only 0.11% (Steele Scott and Timmis, 1984; cf. Timmis and Steele Scott, 1983). Levels of hybridisation of both pTacP6 and pTacB2 to the wheat embryo nuclear genome indicated a considerably greater number of plastome equivalents per haploid genome: 395 and 441, respectively. However, these fragments are unlikely to represent the levels

of homology of other sections of the plastid genome and it appears probable that the extent of this intercompartment transfer may differ between species (Stern and Palmer, 1983). Furthermore the haploid nuclear genome sizes of wheat (17.3 pg) and spinach (0.95 pg) are very different (Bennett and Smith, 1976; Timmis and Steele Scott, 1983).

The mitochondrial genome

Although the percentage hybridisation of pTacP6 and pTacB2 to nDNA were quantified, the levels of hybridisation to the mtDNA were not similarly determined. Lamppa and Bendich (1985) estimated the percentage mtDNA and ptDNA in pea tissues and found that embryos and roots contained approximately equal percentages in the two organelles: 1.5% and 0.5%, respectively, and that in leaf tissue the percentage mtDNA fell to 0.3% or was not detectable. These results give an indication of the potential that exists for cross-hybridisaton of ctDNA fragments to homologous mtDNA sequences, for two plant tissues which are rich in mitochondria; although it is probable that both percentage mtDNA and levels of cross-homology are highly species specific, since the higher plant mitochondrial genome size ranges from 218 kb for Brassica campestris, through 570 kb for maize to as large as 2400 kb in musk melon (Pring and Lonsdale, 1985). Furthermore different sequences with likely homology to ctDNA are not present in equimolar amounts: only the gene for the α subunit, out of the α,β,ε, I and II subunits, of mitochondrial F_1 ATPase has been mapped in maize

mtDNA (Dawson et al., 1986; Isaac et al., 1985), whereas all of the analogous chloroplast F_1 ATPase genes are located in ctDNA (Hird et al., 1986b; Boutry and Chua, 1985; Ohyama et al., 1986); furthermore, only parts of the chloroplast α F_1 ATPase rbcL genes have been identified in maize mtDNA by Lonsdale et al. (1983).

As regards the possible cross-hybridisation of the chloroplast ribosomal fragment P6 it has been found that mitochondrial rRNA shows only 5% observable cross hybridisation, despite being 60-70% conserved relative to ctDNA, whereas the maize mitochondrial copy of chloroplast rRNA was found to be at least 90% homologous (Pring and Lonsdale, 1985). Despite this, when P6 was used to probe Southern blots of PstI restricted total endosperm DNA only one strongly hybridising band was detected, and since the homologous 12 kb mtDNA fragment is overlapped by P6 it is unlikely that PstI digested 16S rRNA fragments from the two genomes would comigrate (see maps Stern and Lonsdale, 1982; Bowman et al., 1981). It was estimated that in the maize mitochondrial genome, the lengths of homologous DNA to both P6 and B2 comprise only 30-50% of those in the plastome: for P6 this represents 6-7 kb compared with 8.4 x 2 kb and for B2 1.86-2.72 compared with 9.2 kb (Stern and Lonsdale, 1982; Lonsdale et al., 1983; Bowman et al., 1981).

From these data an extremely crude estimation of the percentage hybridisation of both P6 and B2 to endosperm mtDNA, relative to ptDNA, was carried out. The following assumptions and approximations were made: mtDNA constitutes 0.50% of total (the percentage in spinach roots where

mitochondria are abundant, Lamppa and Bendich, 1985), ptDNA constitutes 0.91% of total (this thesis section 4.3.6), the wheat mitochondrial genome size is approximately 430 kb (C.J. Leaver, personal communication) and the plastid genome is 135 kb long (the size of the wheat chloroplast plastome, Bowman et al., 1981) and wheat mtDNA contains the same 90-100% homologous regions as found in maize (Stern and Lonsdale, 1982; Lonsdale et al., 1983). Finally, calculations were based on there being only one homologous region per mitochondrial genome and that this hybridises as strongly as the ptDNA sequences. From these data it was estimated that the possible percentage of hybridisation attributed to ptDNA that is due to either P6 or B2 cross hybridisation to mtDNA is from 5.8 to 8.7%.

4.4.5 Percentage ptDNA in wheat endosperm and the effects of grain removal

The percentage ptDNA content of a wide range of plant cells and tissues has now been determined and values range from 0.45% in pea roots (Lamppa and Bendich, 1979a) to 23% in Spinacia oleracea leaves (Steele Scott and Possingham, 1980, 1983). Generally higher percentages have been found in photosynthetic tissues, such as leaves and cotyledons (see Table 4.4.5.1) and the algae Olisthodiscus luteus (4.85%, Cattolico, 1978) and Chlamydomonas reinhardii (10-14%, Whiteway and Lee, 1977). Although potato tubers and spinach cotyledons are exceptions, the former containing between 5% to 22% (Table 4.4.5.1 and Conover and Pryke, 1987) and the

Table 4.4.5.1 Selected data to show the ptDNA and nDNA content of different plant tissues

Plant tissue	Plastid number per cell	% ptDNA	Plastomes per plastid	nDNA per cell (pg)	Reference
Pea:					
leaf	-	8-12	244-174	2-4C	Lamppa and Bendich (1979)
root	-	0.45		2-8C	
embryo	-	1.5		2-4C	
shoot-etiolated	-	1.5		2-16C	
shoot	-	4.3		2-8C	
Spinach:					
leaf: 1 cm	10	7.2	76	1.6	Steele Scott and Possingham (1983)
3 cm	10	8.0	160	3.1	
6-8 cm	17	10.3	110	2.6	
cotyledons:					
7 days dark	-	20	-	2.2	
14 days light	108	22	88	6.9	
expanded leaf:					
0 days	217	19.8	40	6.62	
6 days -N	220	10.0	21	7.96	
Potato:					
leaf	135	7.6	22	6.0	Steele Scott et al. (1984)
stem	-	3.4	-	-	
petiole	-	3.0	-	-	
tuber	40	4.3	195	29.4	
root	-	1.0	-	10.8	
Beet:					
leaf: 2-3 cm	-	7.5	104	2.41	Tymms et al. (1983)
25-30 cm	-	11.4	29	2.67	

latter up to 22% ptDNA.

The estimated 11.1% ptDNA content of wheat leaves (cv. Timmo), determined in this thesis (Table 4.3.6.1), is of a similar order of magnitude to other photosynthetic tissues but is more than two fold greater than the estimated 4.97% for the Mardler wheat variety (Bowman, 1986), although higher estimates of 17% and 13% have been presented by Boffey and Leech (1982) and Dean and Leech (1982a) (see Table 4.4.5.2). Bowman (1986) used scans of agarose gels of PstI digested total leaf DNA to estimate the percentage ctDNA in Triticum and Aegilops species, representing three levels of nuclear ploidy, and found that these were not significantly different. Dean and Leech (1982a), by means of diphenylamine assays of DNase treated chloroplasts obtained estimates of 22% in diploid and tetraploid Triticum species and 13% in T. aestivum.

For tissues where lower percentages have been determined (see Table 4.4.5.1) most experiments used reassociation kinetics, some with ctDNA probes (Lamppa and Bendich, 1979a; Timmis and Steele Scott, 1983) and others with ctDNA fragment probes, such as the rbcL gene (Steele Scott et al., 1984). Since none of these data accounts for background, with the exception of a deduction of 0.07% for cross hybridisation to nDNA in spinach (Timmis and Steele Scott, 1983), they may be significantly overestimated. The range of percentage ptDNA in the developing wheat endosperm was found to be similarly low, ranging from 0.74% or less to 0.91% (Fig. 4.3.6.3).

In order to investigate the potential coding role of

Table 4.4.5.2 Published estimates of the ptDNA and nDNA content of Triticum and Aegilops species

Species and variety	Plastid number per cell	% ptDNA	Plastomes per plastid	nDNA per cell (pg)	Reference
T. aestivum (Timmo):					
leaf	(155)	11.1	(216)	(45.0)	This thesis† and Boffey & Leech (1982)
endosperm:					
10-20 mg	8.5	0.74(0.86)*	393(456)*	68.3	This thesis†
20-30 mg	45.6	0.90	103	81.5	
30-40 mg	54.4	0.91	96	83.7	
40-50 mg	44.9	0.91	124	92.7	
50-60 mg	38.9	0.91	143	72.3	
60-70 mg	32.2	0.91	172	91.5	
70-80 mg	30.5	0.57(0.90)*	114(180)*	92.4	
T. aestivum (Maris Dove)					
leaf: 1-2 (cm from	<50	17.0	810	36.0	Boffey and Leech (1982)
2-3 leaf base)	>50		1020	39.0	
4-5	155		320	45.0	
6-7	155		310	45.0	
T. monococcum (2x)	54	22	-	12.4	Dean and Leech (1982a)
T. dicoccum (4x)	103	22	-	24.2	
T. aestivum (Maris Dove)	133	13	-	34.6	
A. squarrosa (2x)	57	4.9	111	10.2	Bowman (1986)
Ae. speltoides (2x)	62	4.8	114	11.6	
Ae. umbellata (2x)	66	3.8	74	10.1	
T. boeticum (2x)	-	4.4	-	-	
T. dicoccoides (4x)	98	2.8	-	24.5	

Table 4.4.5.2 continued.

Species and variety	Plastid number per cell	% ptDNA	Plastomes per plastids	nDNA per cell (pg)	Reference
Ae. ovata (4x)	94	4.1	102	18.5	Bowman (1986)
T. aestivum (Mardler)	155	4.9	146	34.6	
" (Chinese Spring)	130	4.8	171	34.6	

Tissue, unless stated was taken from expanded leaves of > 20 cm length.

*The different values are presented due to the uncertainty as regards the % ptDNA in very young and mature endosperms (see Fig. 4.3.6.3).
†Cross hybridisation of probe to nDNA was subtracted.

endosperm ptDNA it was of interest to determine whether the cell ptDNA content is related to the number or volume of starch granules. This was investigated by grain removal experiments which resulted in the formation of 40.0% heavier grains (section 2.3.2) which had a 17.6% smaller number and volume of starch granules per cell than normal grains (section 3.3.2). However, no significant difference in the percentage ptDNA was detected in these grains relative to normal grains. Therefore, since the mean endosperm cell ploidy was also not significantly different (section 2.3.4) it would appear that the total cell complement of both nDNA and ptDNA was the same for both grain types; despite a 51% increase in endosperm cell number as a result of grain removal (section 2.3.6). It appears that the ratio of plastid genomes to each nucleus may have remained constant despite a reduction in the number of A type amyloplasts and starch granules per cell which would suggest that the amyloplast genome complement was increased as a result of grain removal. It was considered previously that the rate of starch synthesis in the plastids of these larger grains may be slightly greater than in normal grains (section 3.4.7); if this is correct, the explanation may lie in either the increased nuclear DNA to amyloplast ratio or an increased amyloplast ploidy.

Despite these speculations it is recognised that the experimental variation in the determination of both mean ploidy and percentage ptDNA was not small, making it difficult to conclude that there was no change in either

after grain removal, in parallel with the 17% reduction in starch granule numbers per cell. Furthermore due to the relatively low percentage ptDNA in endosperm tissue, it was considered that this question would be hard to resolve using these techniques (cf. Bowman, 1986).

4.4.6 Plastome copy number in wheat endosperm

Boffey (1985) discusses chloroplast division in relation to the cell cycle and states that chloroplast development is not based on a 'chloroplast cycle' and that the replication of ctDNA is not synchronised with that of nDNA (Rose *et al.*, 1975; Galli *et al.*, 1986) nor are the two directly dependent on each other (Heinhorst *et al.*, 1985). These conclusions were primarily drawn from the investigation of spinach and wheat leaf development where it was determined that initially ctDNA and nDNA synthesis are synchronous and in excess of the rate of plastid division: subsequently, ctDNA replication exceeds that of nDNA and chloroplast division occurs more rapidly, eventually exceeding the rate of ctDNA replication which, with nDNA synthesis, ceases before plastid division (Steele Scott and Possingham, 1980, 1983; Boffey *et al.*, 1979; Boffey and Leech, 1982; Ellis *et al.*, 1983; Lamppa and Bendich, 1980).

Despite the apparent flexibility of ctDNA levels per cell in immature tissue, work on the algae *Olisthodiscus luteus* (Cattolico, 1978) and *Chlamydomonas reinhardii* (Whiteway and Lee, 1977) has indicated that ptDNA levels may be related to nuclear ploidy, a constant ratio being

maintained despite changes in plastid number. This is supported by work on etiolated pea seedlings where nuclear ploidy increased from 2C to 4C yet the percentage ptDNA remained at 1.4% (Lamppa and Bendich, 1979a), as well as by the constancy of the percentage ctDNA in different Triticum and Aegilops species (Bowman, 1986) (Table 4.4.5.2) and, to a lesser extent the parallel investigations of Dean and Leech (1982). However it is again difficult to determine whether the primary determinant of the cell ptDNA complement, within a species or tissue, is the nDNA content or whether cell volume or surface area (see analogous discussion in section 3.4.7), or even plastid volume or area, in conjunction with plastid number, may exert more direct control. As regards the latter, data has been presented which indicates that plastid number alone is not directly coupled to ptDNA content (see section 4.4.7; Cattolico, 1978; Boasson and Laetsch, 1969; Rose et al., 1975). Above all it is evident that the absolute ratio of ptDNA to nuclear volume, cell size, or whatever, for a particular species or tissue (Table 4.4.5.1) or even environment, is ultimately under the control of nuclear genes (Parthier, 1982).

Results presented in this thesis indicate that plastome copy number per endosperm cell was closely related to the mean ploidy throughout grain development, although not at maturation (Fig. 4.3.7.3). These results differ from those for wheat leaves in that leaf ctDNA synthesis continues after nDNA replication has ceased (Boffey et al., 1979). More detailed comparisons are not possible since the endosperm was

not sequentially sectioned from the meristematic layer to the oldest cells, as was possible for the maturing wheat leaf (Boffey et al., 1979; Boffey and Leech, 1982). Therefore all endosperm data represents a mean for cells at a variety of stages of development.

A comparison of the number of plastome copies per cell for mature leaf (33480) and endosperm (5570) cells reveals that these differ by less than do the percentage ptDNAs (estimated from Table 4.4.5.2 data). This is due to the greater mean ploidy of the endosperm relative to the leaf. A trend is visible whereby species having a greater genome weight tend to have a greater number of plastome copies per cell, although exceptions to this exist both between species (Table 4.4.5.1) and, in particular, between tissues (Steele Scott and Possingham, 1983; Steele Scott et al., 1984).

4.4.7 Plastome copy number per endosperm plastid

Rose et al. (1975) found that incubation of spinach leaf discs in the dark inhibited plastid division whereas ctDNA synthesis continued, and Boasson and Laetsch (1969) suggested that chloroplast division is not triggered by ctDNA replication since the inhibition of the latter, in tobacco, by fluorodeoxyuridine, did not cause plastid division to cease. These data and those for spinach and wheat leaf development (Steele Scott and Possingham, 1983; Boffey and Leech, 1982) reveal that chloroplast division and ctDNA synthesis are able to proceed independently.

In endosperm amyloplasts the number of plastome copies

per A type amyloplast increased linearly from 96 in grains of 30-40 mg to 172 in grains of 60-70 mg, in parallel with the period of rapid increase in A type, and therefore amyloplast, modal volume (Fig. 3.3.1.10). It is not clear whether from 10-20 mg to 20-30 mg the true trend is a fall in plastome number per plastid from 393 to 103 or less, as shown (Fig. 4.3.6.4), or a plateau, or a linear increase. For the former the estimated number of A type amyloplasts per cell was 8.5, however if this is not representative of the total plastid number (see sections 3.4.1 and 4.3.6) and the figure is nearer 35 or 74, the shape of the curve may more closely resemble one of the other two alternatives.

For grains of more than 60-70 mg it is considered probable that the percentage ptDNA may decline rapidly (Tables 4.3.5.2 and 4.3.5.3 and Fig. 4.3.6.3), possibly with the onset of maturation (as described by Evers, 1974). If this is correct it indicates preferential loss of plastome copies, relative to the nucleus, towards the cessation of starch synthesis (Figs. 3.3.1.2 and 3.3.1.10). A similar observation was made for spinach leaves where senescence, induced by shading or nitrogen deficiency, was found to result in the loss of 50% of the plastome copies per chloroplast over 6 days (Tymms et al., 1982; Steele Scott and Possingham, 1983).

Whereas it is known that the ptDNA is transcribed in photosynthetically active plastids (Chelm et al., 1977; Rodermel and Bogorad, 1985; Siddell and Ellis, 1975), etioplasts (Miller et al., 1983) and also chromoplasts

(Piechulla et al., 1986), it has not yet been determined whether the plastome is expressed in proplastids or amyloplasts (Aguettaz et al., 1987; Macherel et al., 1986b). Since both wheat endosperm and the enclosed A type amyloplasts are terminally differentiated (Wong and Benedict, 1980), the endosperm ptDNA is not maintained in order that it may be transmitted: the situation is presumably similar in some root tissues. This is in contrast to embryo and cotyledon plastids (Steele Scott and Possingham, 1983; Lamppa and Bendich, 1979a) or even to potato tuber amyloplasts (Steele Scott et al., 1984), since the latter can undergo a transition to become chloroplasts (Conover and Pryke, 1987).

The number of plastome copies per A type amyloplast was similar to the number per chloroplast in pea (Lamppa and Bendich, 1979a), spinach (Steele Scott and Possingham, 1983) and beet (Tymms et al., 1983) leaves, all of which would be expected to be transcriptionally active (Table 4.4.5.1) and was also more than half the estimated plastome number per plastid in wheat leaves of the same cultivar (Table 4.4.5.2). Thus there would appear to be more than sufficient gene copies for ptDNA transcription and translation to occur in the wheat endosperm, especially considering the abundance in leaves of some ctDNA encoded proteins (Ellis, 1979).

CHAPTER 5

PLASTID RNA IN WHEAT ENDOSPERM

5.1 Introduction

It was found that the ptDNA content of wheat endosperm amounts to only 0.91% of total cell DNA or less, whereas the plastome copies per cell may number as many as 5600 (this thesis, sections 4.3.6 and 4.3.7). This number constitutes approximately 16.6% of the equivalent plastome number in wheat leaf cells. Furthermore, the number of plastomes per A type amyloplast appears to be of a comparable order of magnitude to that of chloroplasts in wheat leaf cells, particularly in the youngest and more mature endosperms. It was therefore considered that the amyloplast plastome may be transcriptionally active.

In a preliminary investigation the potential levels of expression of the plastome were determined for grains of 10-20 mg to 70-80 mg which spanned the grain-filling period of development (sections 2.4.6 and 3.3.2). Dot blots of total endosperm RNA samples were probed with the ctDNA 16S ribosomal RNA (rRNA) gene in order to determine whether this gene is transcribed in the endosperm and, if so, whether the relative amounts of transcription vary over the cell division and starch synthesis periods of development.

Subsequently, Southern blots of ctDNA fragments which encompassed the entire wheat plastome were probed with total endosperm RNA from grains of three developmental stages: III,

IV and V, as described by Evers (1974). It was intended that this should reveal which regions of the amyloplast plastome, if any, are transcriptionally active and give an indication of the different levels of expression for the three endosperm stages: these could then be related to the percentage ptDNA content of these endosperms.

5.2 Materials and Methods

All buffers were filter sterilised or autoclaved as described in section 4.2. Glassware was silicon coated and baked at 160°C to denature RNases. All total endosperm RNA was prepared from Batches 3.1 and 4 plants (section 2.2.1).

5.2.1 Total leaf, total endosperm and chloroplast RNA extraction

It was not necessary that RNA used for dot blots and 5' end-labelling should be completely intact. For this reason total leaf and endosperm RNA samples were extracted alongside DNA, from tissue that had been protease digested, phenol and chloroform extracted and centrifuged on CsCl gradients, as described in section 4.2.1. CtRNA was taken from CsCl gradients prepared from lysed chloroplasts, as described in section 4.2.2.

RNA recovery from CsCl gradients

After DNA bands had been removed, CsCl gradient tubes were drained and the RNA recovered from the side, where it had banded vertically (Fig. 4.2; Koller *et al.*, 1982) on ice, RNA samples were dissolved in TE, butan-1-ol extracted five times, and ethanol precipitated in 0.1 vol of 3M sodium acetate and 2.5 vol of ethanol at -20°C, overnight, (section 4.2.1). RNA samples were ethanol precipitated twice more before being dried under vacuum, dissolved in TE and stored at -20°C.

RNA concentration was assayed spectrophotometrically, as described in section 4.2.6.

5.2.2 Polyacrylamide gel electrophoresis of RNA

Total leaf and endosperm RNA samples were electrophoresed through rod gels containing 2.4% w/v acrylamide (Loening, 1967) to separate the four cytoplasmic and plastid ribosomal subunits: 25, 23, 18 and 16S (Reisfeld et al., 1982).

Stock acrylamide was prepared containing 15% w/v acrylamide and 0.75% w/v N,N'-methylenebisacrylamide (both specially purified for electrophoresis, Fisons and BDH, respectively), filtered, and stored in the dark at 4°C. To 1.6 ml of stock acrylamide was added 3.3 ml of BE buffer: 0.12M Tris-HCl, 6 mM EDTA and 0.01 M ammonium acetate, pH 7.8; and 5.1 ml of distilled water. The solution was degassed and 10 μl of TEMED (N,N,N',N'-tetramethylethylenediamine) and 100 μl of freshly prepared 100 mg/ml ammonium persulphate were added to polymerise the acrylamide.

Silicon tubes (10 x 0.4 cm), supported in 'suba-seal' rubber stoppers, were filled with the acrylamide mixture to within 1 cm of the rim and butanol or water was layered on top for 2 h, until the gel had set. Gels were electrophoresed in TAE (TAE is 0.04 M Tris-HCl, 0.033 M sodium acetate, 2 mM EDTA, pH 7.8) and 0.5% w/v SDS; at 60 V for 10 min. Total leaf and endosperm RNA samples, each containing 20-30 μg, were ethanol precipitated and resuspended in 10 μl of TE, 10 μl of Solubilisation buffer: 0.073 M Tris-HCl, 0.066 M sodium acetate, 4 mM EDTA and 20% w/v SDS, pH 7.8; and 10 μl of Stop buffer: 5% w/v SDS, 25%

v/v glycerol, 0.025% w/v bromophenol blue. Samples were loaded onto the gels, electrophoresed at 60 V for 10 min, and then at 1 V (1-2 mA) overnight (or 8 h at 4 mA). A blank tube containing 10 μl of Stop buffer was also run.

Gels were scanned in the tubes at 254 nm, against the blank tube, using a gel scanner (model 1310, MSE), with gel speed of 150 cm/h, and an ISCO UA-5 absorbance-fluorescence monitor, with lamp current at 340 mA. Scans were stopped before reaching the bromophenol blue peak. Data was processed by a LDC/Mitton Roy Cl-10 integrator.

5.2.3 RNA dot blots

Three membrane filters, spotted with chloroplast (ctRNA), total leaf and endosperm RNA were prepared following three procedures: two using glyoxal treated samples (Thomas, 1983) and one using untreated RNA (procedure from Pall Europe Ltd., for Biodyne A membranes).

Filter preparation using glyoxal

Two Biodyne A membrane filters were prepared (P/N BNRG 137, 0.2 μm, Pall), carrying replicate samples: one was presoaked in distilled water, then 20 x SSC and air dried before use (Thomas, 1983); the other was used without presoaking. Crude glyoxal (30% ethanedial in aqueous solution, Fluka) was deionised as described by McMaster and Carmichael (1977) and Carmichael and McMaster (1980). Three 1 ml columns of a mixed bed ion-exchange resin (AG 501-X8 containing a blue pH indicator dye, Bio-Rad) were used, in

sequence, to purify 3 ml of glyoxal which was then stored in full, air-tight, 0.5 ml polypropylene tubes, at -20°C. The pH was checked to be between 5.5 and 6.0, before use: unless deionised, glyoxal can cause extensive degradation of RNA.

RNA samples were not serially diluted before spotting. Samples, each containing 50 μg of RNA (except for ctRNA which contained 5 μg), were mixed with 26.6 μl of 3 M glyoxal and 5 μl of 0.16M sodium phosphate buffer (pH 6.5) and the volume was made to 80 μl with distilled water. The mixtures were incubated at 50°C for 1 h and then cooled on ice.

Filters were clamped for 2 h in a dot blot apparatus, which contained 'O' rings (Minifold SRC-96-D, Schleicher and Schuell), and then removed. This indented the filters, thereby preventing the RNA samples from spreading. Volumes of RNA containing 10, 5, 2.5, 1.3, 0.63 and 0.32 μg were spotted onto each filter, drying the spots with a hair dryer between applications. Filters were baked in a vacuum oven at 90°C for 1 h. Glyoxal was then removed by rinsing the filters in 20mM Tris-HCl, pH 8.0, at 100°C for 10 min: this was necessary to avoid any inhibitory effects of the glyoxal groups on hybridisation (Anderson and Young, 1985).

Filter preparation without glyoxal

RNA samples were diluted in distilled water as above and spotted onto filters. Filters were baked in a vacuum oven, as before.

Hybridisation in formamide to [^{35}S]DNA

DNA formamide buffer: 5 x SSC, 5 x Denhardt's solution (section 4.2.8), 50 mM phosphate buffer, pH 6.5, 0.1% w/v SDS, 250 μg/ml salmon testes DNA (sonicated for 15 min and denatured at 100°C for 10 min) and 50% v/v formamide (Fluka); was sealed into a bag with all three RNA filters, taking 4 ml/100 cm^2 of membrane (section 4.2.9). Formamide was included to promote stronger RNA to DNA hybridisation (Birnstiel et al., 1972). The bag was incubated at 42°C for 14 h before replacing the buffer with 8 ml of DNA formamide buffer containing 20 μCi of [^{35}S]ctDNA fragment P6 (7.7 x 10^8 c.p.m./μg)(section 4.2.7), which had been denatured at 100°C for 10 min. The bag was resealed and incubated, as before, for 42 h.

The filters were then washed following the more stringent procedure described in section 4.2.10, air dried completely, and spotted with radioactive ink. X-ray film (Kodak X-Omat) was exposed to the filters which were then cut up and scintillation counted as described in section 4.2.10.

5.2.4 5' end-labelling of RNA

RNA for hybridisation to Southern blots (section 5.2.5), was radiolabelled using T_4 polynucleotide kinase and [^{32}P]ATP (Bedbrook, 1982). Total leaf and endosperm RNA samples (1.0 μg) were incubated in 5 mM Tris-HCl, 10 mM EDTA and 0.1 mM spermidine (trihydrochloride), pH 9.5; in 34 μl reaction volume at 95°C for 5 min. The mixture was cooled on ice, 20 μl of 0.5 M Tris-HCl, pH 9.5 and 20 μl of 0.1 M $MgCl_2$ were

added and the volume made up to 200 μl with distilled water. To this was added 50 μCi of [γ^{32}P]ATP (>5000 Ci/mmole, in water, Amersham) and 20 units of T_4 polynucleotide kinase (E. coli B, Amersham) and the mixture incubated at 37°C for 30 min.

The sample was immediately phenol extracted once, as described in section 4.2.5, then 0.07 vol of 3 M NaCl and 2.5 vol of ethanol were added and RNA was precipitated at -80°C for 30 min. RNA was pelleted at 13000 x g for 10 min (the radioactive supernatant disposed of), washed in 500 μl of 70% ethanol, at 20°C, repelleted, vacuum dried with a syringe needle inserted through the lid of the microfuge tube, and resuspended in TE, ready for immediate use.

5.2.5 Southern blots of ctDNA fragments and hybridisation to RNA and DNA samples

Southern blots

Plasmid DNA samples containing ctDNA fragments were prepared and the inserts excised using restriction enzymes; the digests were RNase treated and phenol and chloroform extracted, as described in section 4.2.4. DNA was ethanol precipitated at room temperature, to reduce nucleotide contamination, resuspended in TE, and the ctDNA fragment concentration estimated by agarose minigel electrophoresis (section 4.2.6). Sufficient of each ctDNA fragment for six gels (four using 0.7 cm wells and two using 0.35 cm wells) was diluted 4:1 v/v with Loading buffer and the samples electrophoresed through 1% w/v agarose in TBE, containing 0.6

μg/ml ethidium bromide, at 40 V overnight (section 4.2.4). Gels were photographed (section 4.2.4) and then Southern blotted onto Hybond-N (RPN 2020N, Nylon, 0.45 μm, Amersham) (Sealey and Southern, 1983).

DNA was fragmented, before blotting, by placing the gels on a transilluminator (Fotodyne Inc.) and irradiating them with u.v. light at 254 nm for 12 min. Gels were denatured and neutralised as described in section 4.2.8 and DNA was capillary blotted onto Hybond N, which had been prewetted in distilled water, followed by 2 x SSC (see section 4.2.8). After 16 h the membranes were removed and rinsed in 2 x SSC, to remove any adhering agarose, left to air dry, wrapped in cling film, and then placed DNA side down on the u.v. transilluminator for 5 min, to cause DNA to covalently attach to the membrane (Cannon et al., 1985b).

Hybridisation in formamide to [^{32}P]RNA

Total leaf and endosperm RNA samples were 5' end-labelled, as described in section 5.2.4, and filters were hybridised as described by Thomas (1980).

RNA formamide buffer: 5 x SSPE, 50% v/v formamide (Fluka), 5 x Denhardt's solution, 10% w/v dextran sulphate (dextran mol wt. 500K) and 20 μg/ml salmon testes DNA (treated as described in section 5.2.3) and 55 μg/ml Baker's yeast tRNA (transfer RNA, type X-S); was added at 5 ml/100 cm^2 of membrane to four filters in plastic heat-sealed bags which were incubated at 42°C for 8 h with gentle shaking. Formamide was purified before use by being repeatedly frozen

at -20°C, placed at 4°C and the liquid poured away. It was then deionised by stirring for 2 h with 4 g/100 ml of 'Amberlite' MB-3 (monobed resin, BDH, manufactured by Rohm and Haas Co.). RNA formamide buffer was replaced with the same buffer containing 0.8 μg [^{32}P]total RNA (section 5.2.4) at 2 x 10^7 c.p.m./μg and the filters were incubated, as before, for 40 h.

Filters were removed, rinsed in 2 x SSPE, incubated in 2 x 200 ml of 5 x SSPE (section 4.2.8), at 42°C for 2 x 15 min; incubated in 200 ml of 1 x SSPE containing 0.1% w/v SDS, at room temperature for 15 min and then sealed in cling film and autoradiographed, as described in section 4.2.9.

Because filter backgrounds were high the wash stringency was increased. The following wash procedures were used, the filters being autoradiographed after each attempt: (i) 0.1 x SSC containing 0.1% SDS at 50°C for 1 h, (ii) 0.03 x SSC containing 0.1% SDS at room temperature for 1 h and (iii) 0.5 x SSC containing 0.1% SDS in which filters were shaken at 200 r.p.m. at 65°C for 1 h and then rinsed in 0.03 x SSC containing 0.1% SDS at 50°C for 20 min. Finally the filters were treated with either RNase or S_1 nuclease. RNase A (Type IIIA) was prepared at 10 mg/ml and heated to 100°C for 15 min, to denature DNase (Maniatis et al., 1982). One filter was rinsed in 0.1 x SSC and incubated in 10 ml of 0.1 x SSC containing 0.4 mg of heat-treated RNase, at 37°C for 30 min (Gillespie, 1968). It was then rewashed in 0.1 x SSC containing 0.1% SDS and autoradiographed. Another filter was incubated in 50 ml 0.1 x SSC in S_1 nuclease buffer (see

section 4.2.9) containing 600 units of S_1 nuclease and 9 mg of sonicated salmon testes DNA, at 37°C for 2 h. It was then rinsed in 0.1 x SSC containing 0.1% SDS at 65°C for 30 min, before being autoradiographed.

Hybridisation to [^{35}S]DNA

Nuclear and total endosperm DNA samples were nick translated using [^{35}S]dCTPαS, as described in section 4.2.7. Filters were hybridised as follows: DNA prehybridisation buffer: 6 x SSC, 5 x Denhardt's, 0.5% w/v SDS and 20 μg/ml of sonicated, denatured salmon testes DNA; was added at 5 ml/100 cm^2 of membrane to two filters which were incubated in plastic, heat-sealed bags at 65°C for 6 h, with gentle shaking. DNA prehybridisation buffer was replaced with the same buffer containing 100 ng of [^{35}S]DNA at either 8 x 10^7 c.p.m./μg for nuclear DNA or 2.4 x 10^8 c.p.m./μg for total endosperm DNA; the filters were incubated as before for 33 h. They were then rinsed in 2 x SSC and incubated in 2 x 100 ml of 2 x SSC at 65°C for 2 x 15 min, in 100 ml 2 x SSC containing 0.1% SDS at 65°C for 30 min and in 0.1 x SSC at 65°C for 10 min, before being sealed in cling film and autoradiographed, as before.

5.3 Results

5.3.1 Polyacrylamide gel electrophoresis of total leaf and endosperm RNA

Scans of gels of total leaf and endosperm RNA samples, from CsCl gradients, revealed that the RNA was degraded (Fig. 5.3.1), although a large proportion migrated at a fairly high molecular weight. Endosperm RNA appeared to be less degraded than leaf, possibly due to a lower endogenous RNase content in endosperm tissue.

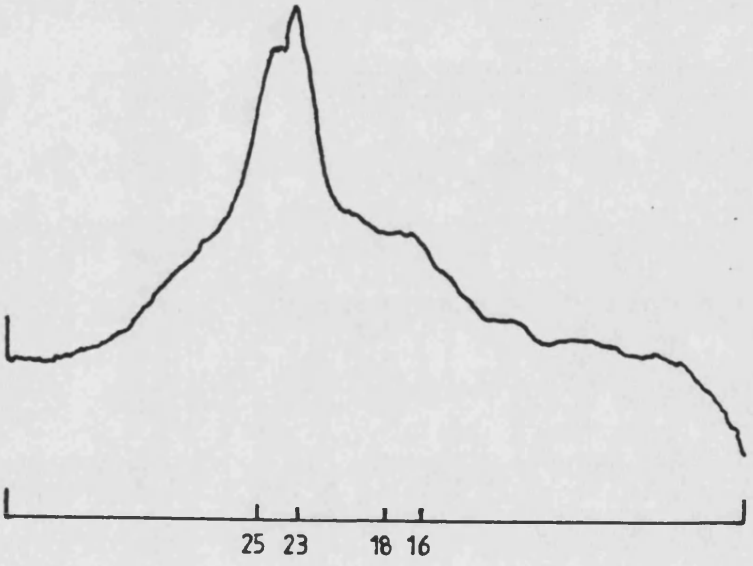


Figure 5.3.1 Absorbance scan at 254 nm of total endosperm RNA (from A grains at 17 d.p.a.) electrophoresed through a polyacrylamide tube gel. Mean 25, 23, 18 and 16S rRNA peak positions for three standard samples are shown.

Because the samples were degraded it was not possible to determine whether plastid rRNA was present in the endosperm

tissues (cf. Reisfeld et al., 1982); as the 23S and 16S plastid rRNA absorbance peaks could not be distinguished. Rather than attempt to extract totally intact rRNA, especially considering the low percentage ptDNA found in endosperm, it was decided that dot blots of total endosperm RNA should be probed with P6, to look for expression of the plastid ribosomal genes.

5.3.2 RNA dot blots probed with P6

Denaturation of RNA

Techniques described for RNA dot blots usually suggest that, for efficient binding to filters, RNA should be denatured using glyoxal (Thomas, 1983). This is because RNA, although largely single stranded, also contains double stranded regions: these become single stranded when at acid or neutral pH glyoxal binds covalently to guanine residues (Anderson and Young, 1985). Despite this, not all published procedures suggest denaturing RNA to be loaded onto membrane filters (procedure from Pall Europe Ltd. for Biodyne A membranes). To assess these procedures, RNA dot blots were prepared following three protocols, as described in section 5.2.3.

Visual comparison of autoradiographs revealed few differences between filters spotted with not denatured and denatured RNA (Fig. 5.3.2.1(i) and (ii)). The mean c.p.m. per μg of RNA are presented for those samples which hybridised most (Table 5.3.2.1).

Highest levels of hybridisation were attained when the

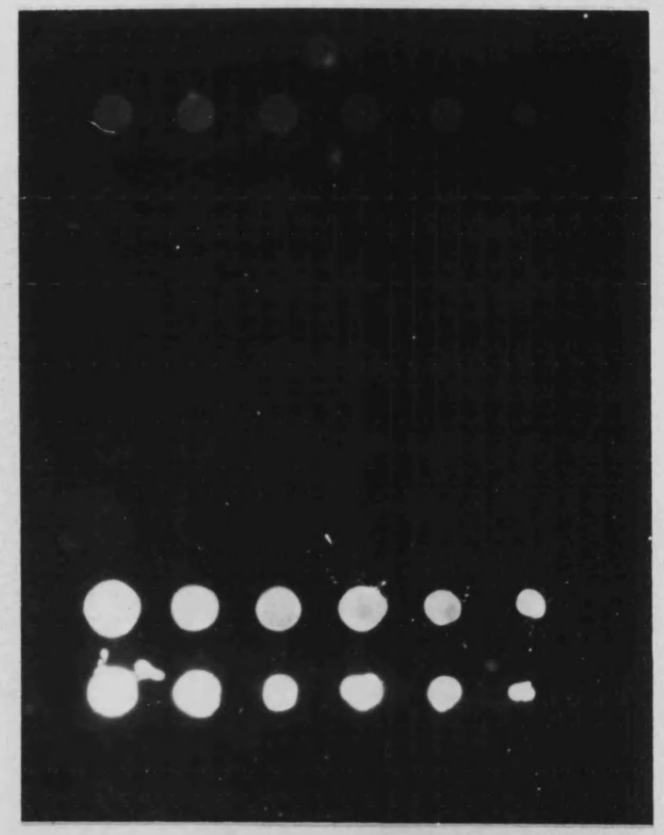
Figure 5.3.2.1 RNA dot blots probed with P6:
(i) RNA not glyoxal treated
(ii) Filter prewetted in 20 x SSC. RNA glyoxal treated.

Key

1. Endosperms at 7 d.p.a. 17.6 to 0.55 μg
2. " " 17 " 8.7 to 0.27 μg
3. " " 20 " 9.1 to 0.28 μg
4. " " 23 " 8.8 to 0.28 μg
5. " " 26 " 8.8 to 0.28 μg
6. " " 29 " 8.8 to 0.28 μg
7. Wheat leaves at $4\frac{1}{2}$ wks 15.2 to 0.48 μg
8. Chloroplasts prepared from wheat leaves 0.90 to 0.03 μg

RNA came from A grain endosperms (Batch 3.1)

(i)



(ii)

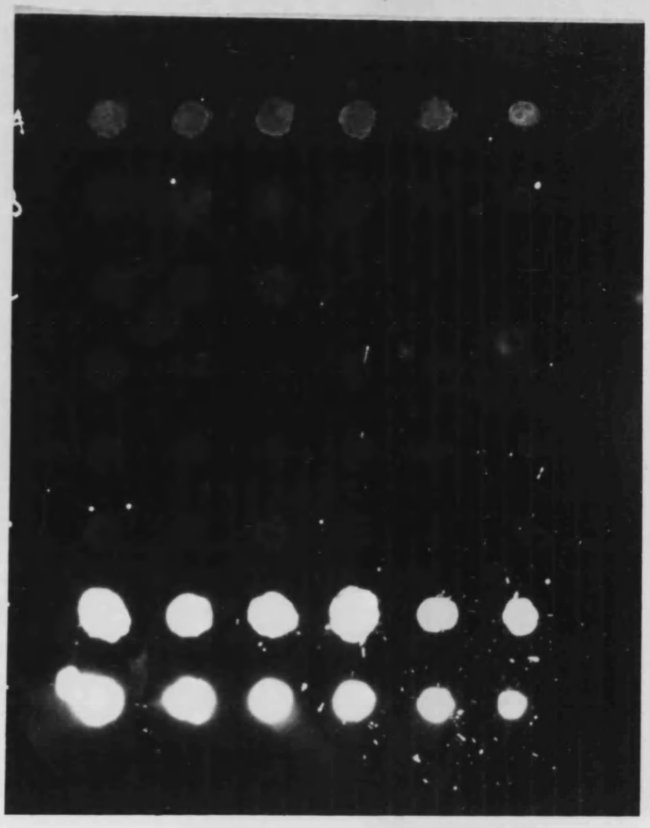


Table 5.3.2.1 Results of RNA dot blots probed with P6

Source of filter bound RNA	Filter c.p.m. per µg (i) RNA not glyoxal treated	(ii) Filter prewashed in 20 x SSC. RNA glyoxal treated	(iii) RNA glyoxal treated	$\bar{x}$
Endosperms at 7 d.p.a.	22.6	20.6	20.8	21.3
Endosperms at 17 d.p.a.	8.0	n.d.	44.1	26.1
Leaves	641	1071	1264	992
Chloroplasts	36487	38149	52009	42215

n.d. = counts not detectable above background

Results are means of six dots on each filter.

RNA was denatured and spotted onto a Biodyne A membrane that had not been prewetted in 20 x SSC. This may have been due to an unequal distribution of probe during hybridisation, since background counts for this filter were also slightly higher. The filter with the lowest levels of background counts was that bearing not denatured RNA.

Levels of hybridisation

Curves of c.p.m. per spot against amount of RNA loaded were linear for only a restricted range of filter bound sequences (Fig. 5.3.2.2). This lack of linearity indicates that at higher spot concentrations the probe concentration was limiting the rate of hybridisation. When homologous, filter bound sequences are in excess over the probe it is the rate of probe diffusion, rather than the nucleation event, which determines hybridisation rates (Anderson and Young, 1985). For long incubations, as the probe concentration falls, due to both hybridisation and reassociation, hybridisation to spots bearing smaller amounts of RNA also starts to be restricted. Since these filters were incubated for 42 h and because the endosperm RNA contained vastly fewer complementary sequences than the chloroplast and leaf RNA (Table 5.3.2.1), the endosperm samples hybridised more slowly. As a result, these curves were the least linear. In fact curves would probably have reached a plateau sooner if it were not for the fact that spot dimensions were still increasing. The results presented in Fig. 5.3.2.2 were for non-denatured RNA, however the same trends were evident using

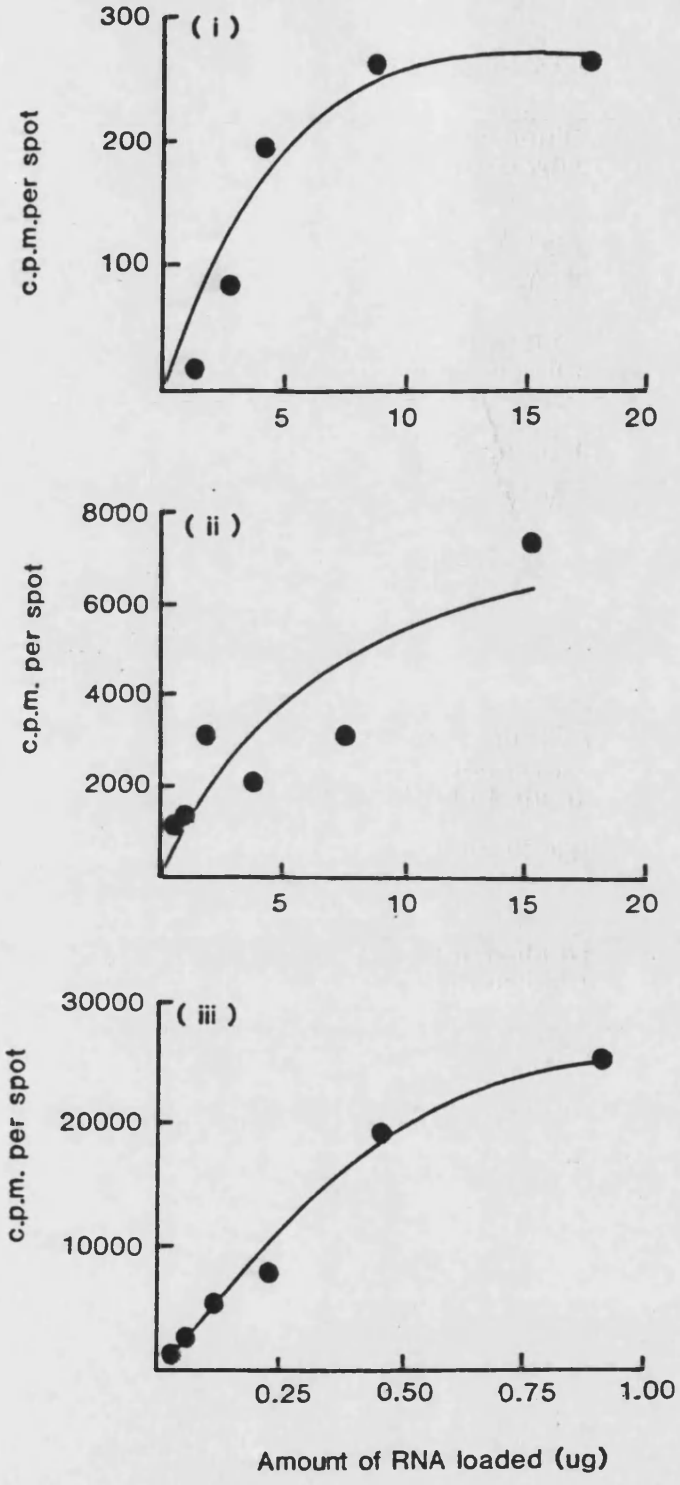


Figure 5.3.2.2 RNA dot blots probed with P6; counts detected per spot for:
(i) total endosperm RNA at 7 d.p.a
(ii) total leaf RNA at $4\frac{1}{2}$ wks.
(iii) chloroplast RNA

denatured RNA.

Comparison of the linear portion of each curve, indicated that the percentage of total leaf RNA showing homology to P6, relative to ctRNA, was approximately 2.69%, whereas in endosperms at 7 d.p.a. the figure was nearer 0.09% (Table 5.3.2.2). Since P6 contains the genes for the 16S and part of the 23S ribosomal subunits (Fig. 4.3.2.1) it was expected that it would be highly expressed in plastids actively transcribing and translating ptDNA. It is perhaps surprising that these percentages were not higher, particularly for the leaf where the percentage ptDNA was estimated to be as much as 11.1% (section 4.3.6). In fact, the results may be quite low because the filter bound total leaf and endosperm RNA samples were partially degraded (section 5.3.1). It is thought that this could reduce levels of hybridisation (section 4.3.2). The ctRNA may have been relatively intact since there was no protease digestion step in the preparation procedure (as there was for total RNA extractions).

Because levels of hybridisation of P6 to total endosperm RNA were low, densitometric scans of autoradiographs were used to compare between endosperm samples (Table 5.3.2.3). These indicated that total levels of expression of the rRNA genes did not differ by more than two fold throughout the period of rapid growth of A grain endosperms, from 7 to 29 d.p.a. The percentage ptDNA in endosperm was also found to

Table 5.3.2.2 Comparison of the amounts of P6 hybridising to chloroplast, total endosperm and leaf RNA

Source of filter bound RNA	Initial rate in c.p.m./µg	Percentage hybridising relative to ctRNA	Percentage hybridising relative to total leaf RNA
Endosperms at 7 d.p.a.	40	0.091	3.38
Leaves at $4\frac{1}{2}$ wks	1185	2.69	-
Chloroplasts	44000	-	-

Table 5.3.2.3 Results of RNA dot blots probed with P6

Source of filter bound RNA	Filter sum peak height per µg (i) RNA not glyoxal treated	(ii) Filter prewashed in 20 x SSC. RNA glyoxal treated	(iii) RNA glyoxal treated	$\bar{x}$ (σ_{n-1})
Endosperms at:				
7 d.p.a.	6.9	16.0	12.7	11.8 (4.6)
17 "	3.7	10.3	12.1	8.7 (4.4)
20 "	8.1	14.4	9.9	10.8 (3.2)
23 "	3.2	5.2	7.1	5.2 (1.9)
26 "	2.3	8.2	12.2	7.5 (5.0)
29 "	7.7	11.1	13.4	10.8 (2.9)

Results are means of up to six dots per filter.

remain quite constant over this period (section 4.3.6). Scans of endosperm RNA dot blots could not be compared with those for leaf and chloroplast RNA because the latter both hybridised so strongly that the film response was no longer linear (section 4.3.5).

5.3.3 Southern blots of ctDNA probed with total endosperm DNA and nDNA

Fragment sizes of digested pTac plasmids, pBR322 derivatives containing ctDNA inserts, were compared with those previously published for the same fragments (Fig. 5.3.3.2(i) and Table 5.3.3.1), in order to determine whether plasmids were fully restricted. Size was estimated by comparison with HindIII digested lambda DNA. Fragments measuring approximately 4.36 kb more than the expected size were assumed to be linear pTac molecules (lanes 12 and 13). Some molecules of pTacS3a, pTacS3b and pTacS8 were not restricted (lanes 10, 12 and 13).

Southern blots of these plasmids hybridised poorly to total endosperm DNA (Fig. 5.3.3.1(ii)). This was in part due to the low percentage of the total radiolabelled probe represented by each fragment, since ptDNA was estimated to comprise only 0.85% for A grain endosperms at 20 d.p.a. (section 4.3.6), but probably also because experimental conditions were not optimal (the ^{35}S[dCTP] used was several months old). Fragments which were found to hybridise were: B8, B9, P3, P4, P7, B2, B3, S3a, P6 and S8 and those which hybridised very weakly were: B10, P10, S3b. Those which

Table 5.3.3.1 Comparison of expected and estimated fragment sizes from pTac plasmids after restriction enzyme digestion

Lane number	Plasmid	Fragment sizes expected*	Fragment sizes estimated**
1	pTacB8+9	5.6 5.3	- 4.91 4.66
2	B10-18	4.8 3.25 2.30?	4.67 3.19 2.21 1.50
3	P10	5.2	5.20
4	P3	14.5	15.49
5	P4	12.6	14.45
6	P7	8.1	8.85
7	B2	9.6	10.00
8	B2-15	9.6 4.18 3.25 ?	11.48 4.15 3.23 1.58
9	B11-3	8.0 4.55 1.68	9.77 4.68 1.86
10	S3a	14.2	- 15.85
11	P6	8.4	8.81
12	S8	4.5	- - 10.0 4.15
13	S3b	13.6	- - 18.2
14	HindIII digested lambda DNA		

*Source: Bowman, Koller, Delius and Dyer, 1981.

**Excised, linear pBR322, at 4.36 kb, was not included. Fragments marked - denote unrestricted plasmid DNA.

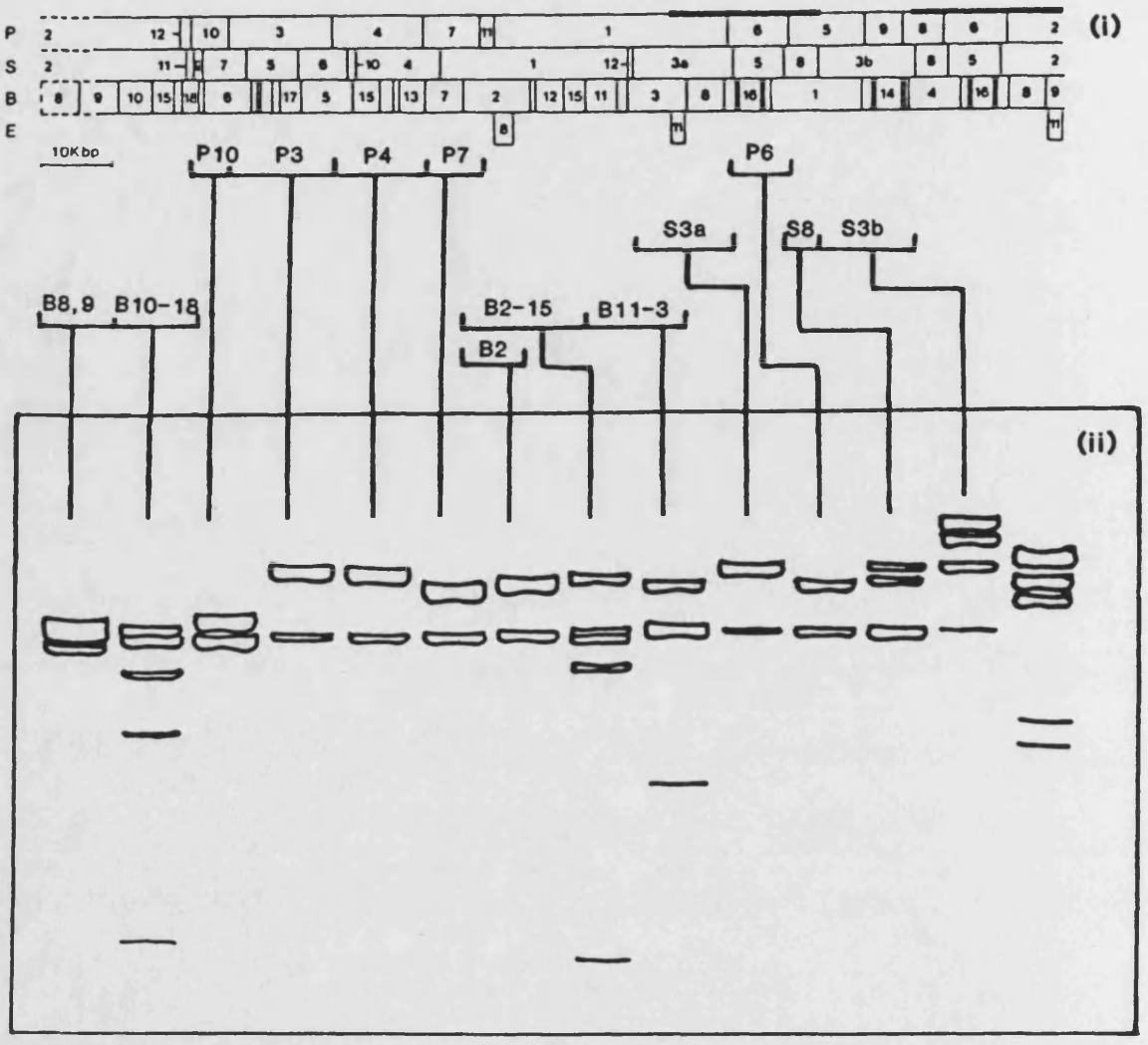
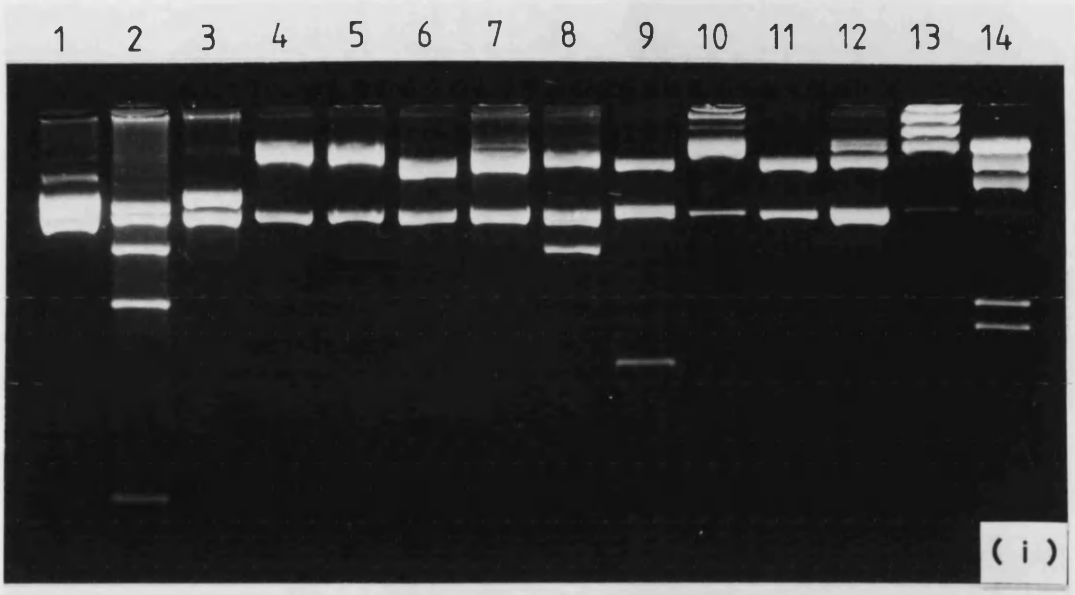
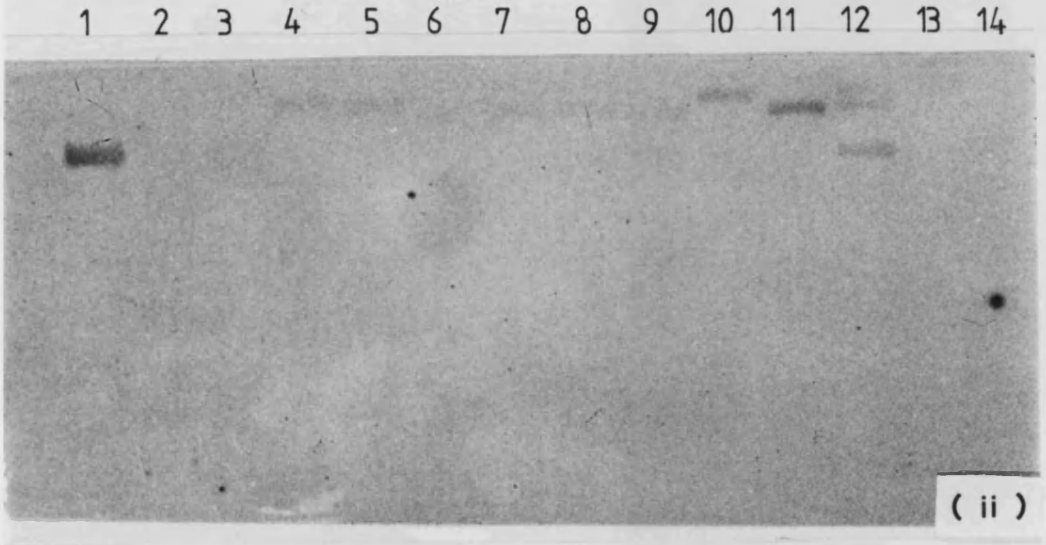
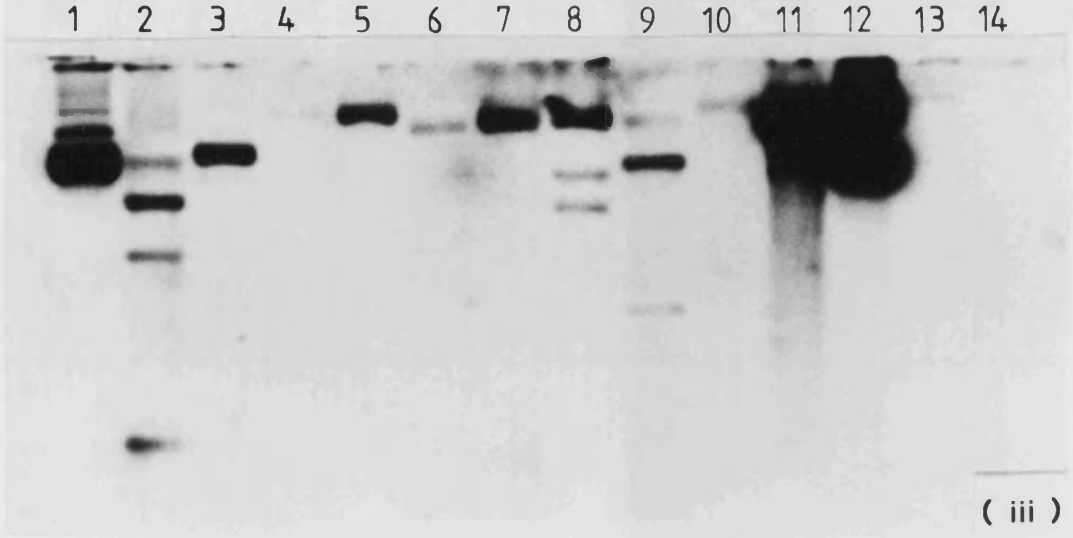


Figure 5.3.3.1 (above) Key to Fig. 5.3.3.2:
(i) restriction map of wheat ctDNA (kindly provided by Cathy Bowman at P.B.I., Cambridge): P indicates PstI inserts, S indicates SalI inserts, B indicates BamHI inserts, E indicates EcoRI inserts, and (ii) diagram of Fig. 5.3.3.3(i).

Figure 5.3.3.2 (facing page). Southern blots of restricted pTac plasmids containing ctDNA inserts as described (Fig. 5.3.3.1 and Table 5.3.3.1):
(i) plasmid fragments electrophoresed through an 1.0% agarose gel,
(ii) Southern blot of the same gel probed with total DNA from A grain endosperms at 21 d.p.a., and
(iii) Southern blot of a similar gel probed with total RNA from wheat leaves.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
(i)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
(ii)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
(iii)

could not be detected in this experiment included: B15, B18 B12 and B11. From these results it was evident that there is widespread homology between wheat chloroplast and total endosperm DNA.

A simlar filter probed with an equivalent amount of HpaII restricted nDNA (section 4.3.5) could not be detected hybridising to any of the fragments with the possible exception of P6: this indicates that there are proportionately fewer sequences homologous to ctDNA present in the nuclear genome of wheat alone (see also section 4.3.2).

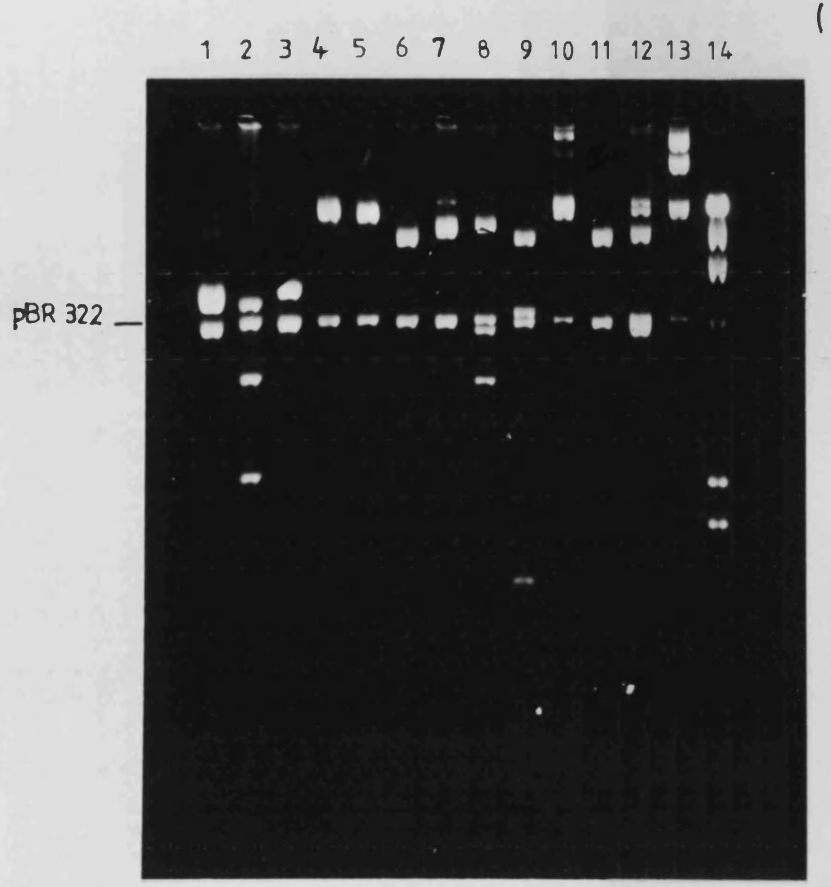
5.3.4 Southern blots probed with total leaf and endosperm RNA

Southern blots, similar to those in section 5.3.3, were probed with total leaf and endosperm RNA (Figs. 5.3.3.1(iii), 5.3.4.1 and 5.3.4.2). As expected, from the RNA dot blots (section 5.3.2), amounts of hybridisation of ctDNA fragments to leaf RNA were far greater than to endosperm RNA. P6 hybridised quite strongly to both total leaf and endosperm RNA (lane 11 in all three figures), although the frequency of homologous sequences in these RNAs may be as low as 2.7% and 0.1%, respectively, relative to ctRNA (at 100%) (section 5.3.2). The only other fragment to hybridise quite strongly to total endosperm RNA was S8, the fragment bearing the genes for the 23, 4.5 and 5S ribosomal RNAs (lane 12, Figs. 5.3.4.1(ii) and 5.3.4.2(ii)) (for map see Fig. 4.3.2.1).

Endosperm RNA probes were prepared from grains in stages III, IV and V of development (Evers, 1974): (i) immature

Figure 5.3.4.1 Southern blot of restricted pTac plasmids containing ctDNA inserts as described (Fig. 5.3.3.1 and Table 5.3.3.1):
(i) plasmid fragments electrophoresed through an 1.0% agarose gel.
(ii) Southern blot of the same gel probed with total RNA from A grain endosperms at $9\frac{1}{2}$-10 d.p.a.(Batch 4).

(i)



(ii)

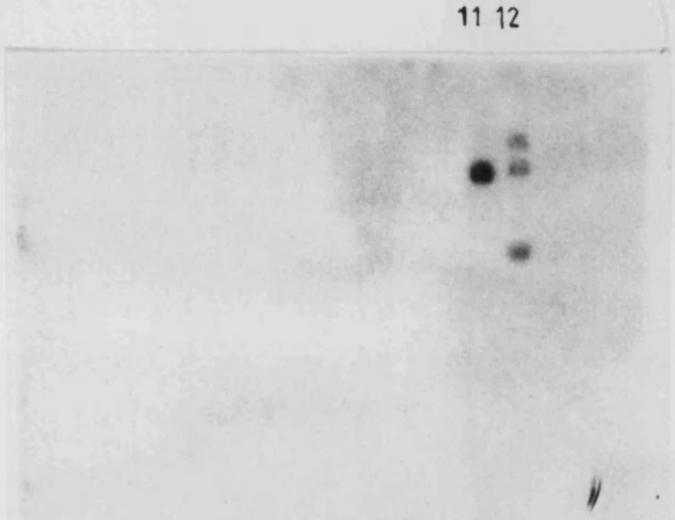
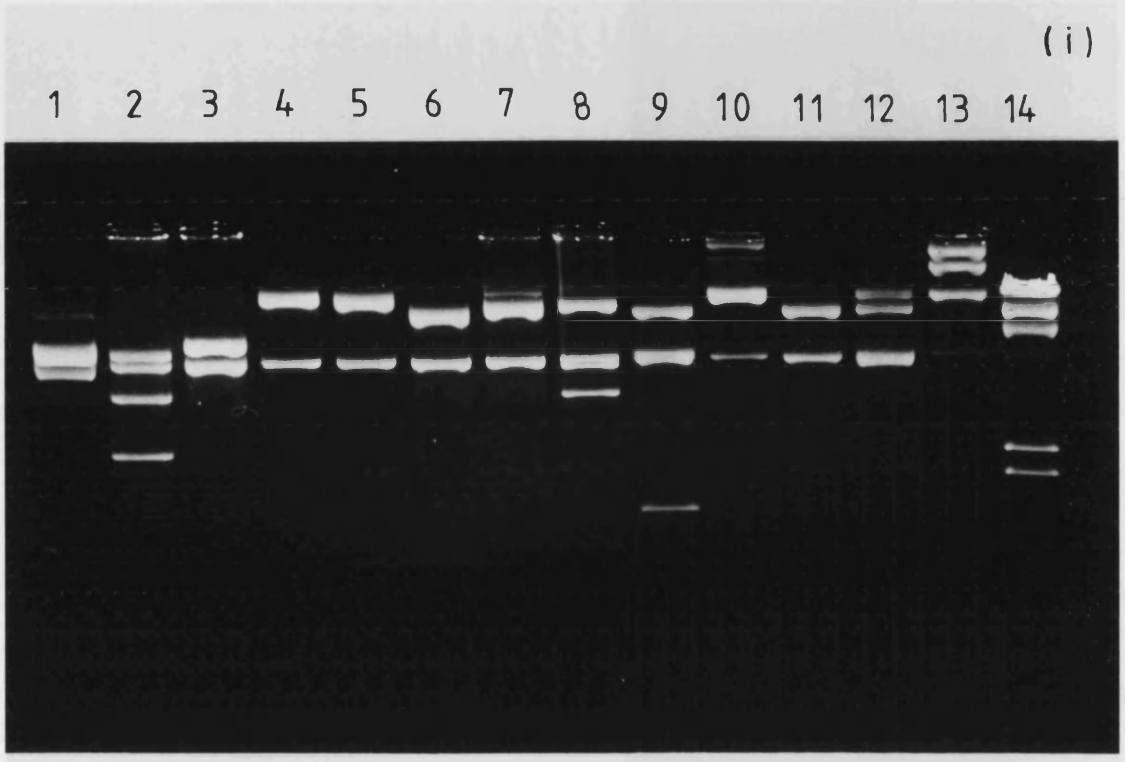
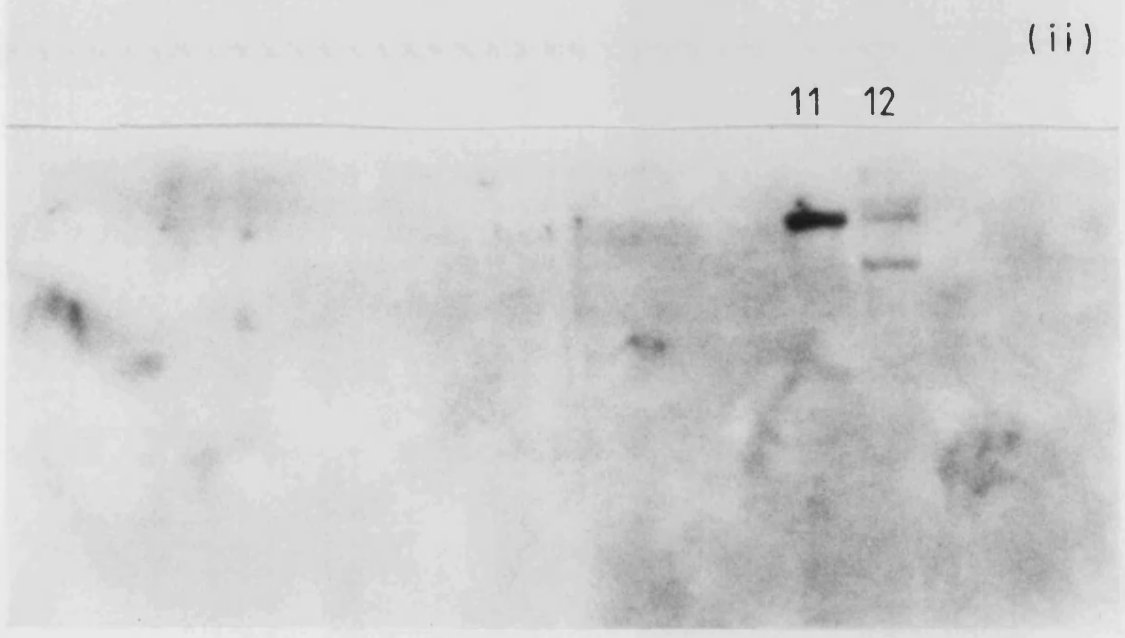


Figure 5.3.4.2 Southern blot of restricted pTac plasmids containing ctDNA inserts as described (Fig. 5.3.3.1 and Table 5.3.3.1):
(i) plasmid fragments electrophoresed through an 1.0% agarose gel.
(ii) Southern blot of the same gel probed with total RNA from A grain endosperms at 29 d.p.a. (Batch 3.1).


(i)
1 2 3 4 5 6 7 8 9 10 11 12 13 14


(ii)
11 12

tissue where both starch granule and cell number were rapidly increasing, from A grains at $9\frac{1}{2}$-10 d.p.a. (14.5-18.7 mg) (Fig. 5.3.4.1(ii)), (ii) developing grains where B type starch granules were being rapidly initiated, from A grains at 20 d.p.a. (46.5-54.0 mg)(results not presented, but indistinguishable from Fig. 5.3.4.1) and (iii) grains approaching maturity where starch synthesis had slowed, from A grains at 29 d.p.a. (68-73 mg) (Fig. 5.3.4.2). There were no large visible differences between endosperm RNA probes with respect to their levels of hybridisation to P6 and S8.

If there was any hybridisation of endosperm RNA to fragments other than P6 and S8 it was not evident. Filter backgrounds were higher than anticipated, possibly in part due to the presence of dextran sulphate and a fairly high concentration of [^{32}P]RNA in the Hybridisation buffer (Anderson and Young, 1985): in addition, [^{32}P]RNA was only purified by ethanol precipitation. Unfortunately, because of these backgrounds, low abundance endosperm plastid RNAs, if present, were not detected. Increasing the wash stringency reduced filter backgrounds: results presented here were achieved by washing the filters in 0.1 x SSC containing 0.1% SDS at 50°C, with shaking. However beyond this neither RNase (Gillespie, 1968) nor S_1 nuclease (Anderson and Young, 1985) treatments of the filters improved results. The former succeeded in removing all detectable counts from the filter, the latter also appeared to reduce hybridised counts as well as filter backgrounds.

The filter probed with total leaf RNA yielded more

information as regards ptDNA expression (Fig. 5.3.3.2(iii)). Fragments hybridising most were P6 and S8 (lanes 11 and 12), presumably due to an abundance of plastid ribosomes in young, green leaves. Either or both of fragments B8 and B9 (lane 1) also hybridised very strongly, although this was in part due to the large amounts of ptDNA loaded on the gel. Included in B9 is the photosystem II (PsII) gene psbA and the $tRNA^{His}$ gene (Quigley and Weil, 1985), from which extends the inverted repeat (for map see Fig. 4.3.2.1). Other PsII genes include psbD on fragment B15 (lane 2), psbC on both B15 and a smaller BamHI fragment (lane 2), psbF and psbE on B12 (lane 8) and psbB and psbH on B11 (lane 9): all of these fragments hybridised reasonably strongly, with psbF and psbE giving the weakest signal. Similarly, transcripts from the fragments P4 (lane 5), which includes the PsI genes, psaAB and the ATP synthase genes atpH, atpF and atpA; B2 (lanes 7 and 8), which includes the rbcL gene for the large subunit of ribulose bisphosphate carboxylase oxygenase; the ATP synthase genes atpB and atpE and the gene petA; and B11 and an adjacent small BamHI fragment (lane 9), which include the genes petB and petD, appear to be quite abundant. Fragments showing only very low levels of expression in young wheat leaves included P3, P7, B3, S3a and S3b (lanes 4,6,9,10 and 13), which together comprise 30.7% of the genome. In addition B10, B12 and B15 (lanes 2 and 8) were not heavily transcribed.

5.4 Discussion

5.4.1 The amyloplast plastome of wheat endosperm

Macherel et al. (1985) successfully isolated amyloplast DNA from sycamore cell cultures and demonstrated, by restriction enzyme digestion and Southern blot analysis using maize ctDNA, that this DNA was seemingly homologous to ctDNA. Similar results were obtained from analogous experiments performed on tomato fruit chromoplast DNA (Iwatsuki et al., 1985). Since the plastids of both cultured cells and tomato fruit contain the potential to undergo a plastid transition either from or to chloroplasts (section 4.4.2) it would appear highly probable that the plastomes are identical, within these tissues. In the endosperm, the only transition that occurs is from proplastid to amyloplast (Parker, 1985), therefore the majority, if not all, of the chloroplast specific genes are likely to remain redundant, if present.

In this thesis it was determined that total endosperm DNA shows widespread homology to wheat ctDNA (Fig. 5.3.3.2(ii)) under stringent wash conditions, which is a further indication that the two plastid genomes are similar (see also section 4.4.2) and that major deletions have not occurred within the endosperm plastome (Day and Ellis, 1984; 1985). As a control a Southern blot of the same ctDNA fragments was hybridised to HpaII restricted nDNA. This did not confirm that there is extensive, strong, cross-homology between the nuclear and plasmid genomes in wheat (see section 4.4.4 and cf. Timmis and Steele Scott, 1988).

5.4.2 Levels of 16S rRNA in wheat endosperm

The presence of sequences homologous to the ctDNA fragment P6, which includes the 16S rRNA gene (Fig. 4.3.2.1), in total wheat endosperm RNA, as well as in wheat leaf RNA (Fig. 5.3.2.1), was an indication that the amyloplast plastome was being transcribed, albeit at a low level.

It has already been discussed (section 4.4.4) that the mitochondrial genome of maize contains a 12 kbp region of at least 90% homology to the 16S rRNA region of the plastome (Stern and Lonsdale, 1982). It is therefore possible that the total cell RNA of maize tissue may contain 16S rRNA transcripts which originate from the mitochondria (Lonsdale et al., 1983). However it appears unlikely that the mitochondrial genome would produce sufficient transcripts to hybridise to the extent found in the total endosperm RNA (see Table 5.4.2 and discussion in section 4.4.4). In addition, Northern blot analysis has revealed homology to the 16S rRNA gene in total RNA of sycamore suspension culture cells (Macherel et al., 1986b), which gives support to the probable location of these transcripts in the amyloplast.

Although wheat nDNA was found to contain sequences homologous to P6 (section 4.3.6), it is not likely that ctDNA encoded genes will be transcribed in the nuclear genome, furthermore Steele Scott and Timmis (1984) claim that cross homology of ctDNA fragments is usually to heavily methylated regions of the nDNA which are likely to be less transcriptionally active.

Table 5.4.2 Relative levels of expression of the 16S rRNA genes in leaf and endosperm plastids

Source of DNA/RNA	% ptDNA*	% transcription** of 16S rRNA per plastome relative to in leaf	Plastome* copies per plastid	%16S rRNA** transcription per amyloplast relative to the chloroplast
Leaf ($4\frac{1}{2}$ wks)	11.1	100	216	100
Endosperms at:				
7 d.p.a. (15.5-21.0 mg)	0.74	50.7	393	92.2
17 d.p.a. (39.0-46.0 mg)	0.91	30.4	124	17.5
20 d.p.a. (46.5-54.0 mg)	0.91	37.6	143	24.9
23 d.p.a. (54.5-61.5 mg)	0.91	18.2	143	12.0
26 d.p.a. (62.0-68.0 mg)	0.91	26.2	172	20.8
29 d.p.a. (68.0-73.0 mg)	0.57	60.6	80	20.1

*Data from Table 4.4.5.2

**Calculated from data in Tables 5.3.2.2 and 5.3.2.3.

Since a large proportion of the chloroplast genome is known to encode proteins involved in photosynthesis (Shinozaki et al., 1986; Ohyama et al., 1986), it was considered unlikely that the amyloplast genome would be as transcriptionally active as the chloroplast. In fact, it was found that relative to leaf ctRNA the fraction of 16S rRNA sequences in total endosperm RNA constituted 3.3% or less (Table 5.3.2.2) whereas a comparison of the DNA levels revealed that ptDNA in the endosperm relative to the leaf, as a percentage of total, was greater at 8.1% (Table 4.4.5.3). There was no evidence of a direct relationship between gene dosage and transcript level during endosperm development since the level of 16S rRNA hybridisation per plastome ranged from 18.2% to 60.6%, relative to 100% in the leaf. This contrasts with the results of Aguettaz et al. (1987) where variation in transcript hybridisation to 16S rDNA and rbcL gene probes were correlated with changes in cell ptDNA levels during an amyloplast to chloroplast transition in spinach cell suspensions. However, Borroto and Dure (1986) found that although ctDNA per cell in cotton cotyledons increased during germination, this increase was less than the increase in RNA transcripts from the ATPase, rDNA and rbcL genes.

Based on the estimated number of plastomes per plastid values for the relative levels of transcripts of 16S rRNA per amyloplast relative to the chloroplast were determined (Table 5.4.2): these ranged from 12% to 25% throughout grain filling, with a higher percentage in plastids of the youngest endosperms. From these results it appears that transcripts

from the 16S rRNA gene may be present in sufficient amounts for the amyloplasts to contain functional ribosomes. It is also evident that there was no significant increase in levels of these transcripts during the period of rapid starch synthesis (Fig. 3.3.1.2) for grains of more than 40-50 mg. Levels of the 16S rRNA transcript per plastid in young endosperm tissue appeared to be considerably greater than in the more mature endosperm, however as discussed previously (section 4.4.7), this may be based on an erroneously low estimate of the plastid number.

It has been found that the concentration of specific transcripts can vary with the stage of plastid development and in a tissue-specific manner (Mullet and Klein, 1987; Rodermel and Bogorad, 1985). In barley, and also in other plants, the levels of the psbA mRNA have been reported to increase significantly on illumination (Kreuz et al., 1986) whereas the transcripts of the rbcL, psaA and psaB genes were found to decline during plastid maturation (Mullet and Klein, 1987). Mullet and Klein (1987) present evidence that changes in the levels of some barley plastid mRNAs (rbcL, psaA-psaB) were due to altered transcription but that for others (16S rRNA, psbA) transcript number was primarily determined by post-transcriptional events, affecting RNA stability. Over a five day period, in non-illuminated barley seedlings, the plastid content of 16S rRNA transcripts remained constant despite a 90% reduction in transcription of this gene: the authors propose that the stability of the 16S rRNA may be attributed to the ribosomal proteins of the 70S ribsome and

that transcription of the 16S rRNA gene is controlled by a different mechanism to that of the protein coding genes.

In the light of these observations, it is possible that the constancy of transcript levels per amyloplast in grains of 40-50 mg and upwards, where division of A type amyloplsts has ceased (section 3.3.1(d)), may be attributed to the stability of this RNA rather than to continued transcription. However for this to be possible considerable transcription must have occurred in the young endosperm, presumably under the control of a light independent promoter.

5.4.3 Lack of evidence for transcription of protein coding genes in wheat amyloplasts

Southern blots of ctDNA fragments probed with total endosperm RNA revealed the presence of transcripts with homology to the fragments P6 and S8, which bear the genes for the 16, 23, 4.5 and 5S rRNAs (Figs. 5.3.3.3 and 5.3.3.4: map is Fig. 4.3.2.1). In the previous section it was discussed that the level of 16S rRNA in the amyloplast, although low relative to the chloroplast, may be sufficient for these organelles to contain functional ribosomes. The presence of transcripts to at least one of the other rRNA genes helps to validate this. However, due to the relatively low proportions of total endosperm RNA that these rRNA transcripts comprise (Fig. 5.3.2.1) and because the ratio of individual mRNA to rRNA transcripts is usually low (see Fig. 5.3.3.2, lanes 11 and 12 compared with other lanes, also Borroto and Dure, 1986; Link, 1984), these results were

insufficiently sensitive to detect the presence, or verify the absence, of other RNA transcripts. This was probably made particularly difficult due to the abundance of nuclear encoded mRNA produced in maturing endosperms which encode the prolamin wheat storage proteins (Bartels and Thompson, 1986; Pernollet and Vaillant, 1984; Greene, 1983).

Other studies on amyloplasts from sycamore cell suspension cultures (Macherel et al., 1986b) and potato tuber amyloplasts (Lobov and Bondar, 1977) have been equally unrevealing, although it would appear that these tissues contain sufficient ptDNA for the plastids to be transcriptionally active, as was determined for endosperm amyloplasts (section 4.4.7).

From studies on etioplasts (Link, 1984; Mullet and Klein, 1987; Seyer and Lescure, 1984; Obokata, 1984; Jenkins et al., 1983; Miller et al., 1983), cotton cotyledon plastids (Borroto and Dure, 1986) and chromoplasts (Piechulla et al., 1986) it is apparent that plastids other than chloroplasts may be both transcriptionally and translationally active, particularly in the synthesis of rbcL mRNA and 16S rRNA. Furthermore, a number of reports have detected low levels of the rbcL or 16S rRNA transcripts in leucoplasts or amyloplasts from suspension cultured cells (Richter, 1984; Aguettaz et al., 1987; see also Wong and Benedict, 1980) and the psbA transcript has been detected in tomato roots (Piechulla et al., 1986); although in the former the distinction between these plastids and immature etioplasts is unlikely to be great (Kirk and Tilney-Bassett, 1978). From

this it appears that transcriptional activity in the endosperm amyloplast cannot be ruled out; particularly in younger endosperms (Table 5.4.2).

CHAPTER 6

DISCUSSION

Grain yield potential

The results of chapters 2 and 3 of this thesis reveal that wheat grains (cv Timmo) of the third floret in each spikelet contain an unexploited capacity which amounts to approximately 44% and 40% of grain fresh and dry weight, respectively, and 53% and 34% in terms of endosperm cell number and starch granule content. If the constraints which limit the size of the third floret grain were determined, it may be possible for the yield of distal, and possibly also basal, grains to be considerably improved: by either plant breeding or genetic manipulation. This is based on the assumption that endosperm cell division and, in particular, cell growth are rarely limited by an inadequate assimilate supply, in vivo (Jenner and Rathjen, 1972a,b; Singh and Jenner, 1982b; 1984) except under fairly severe environmental stress (section 1.3(iii) and Table 3.4). However, as described by Trewavas (1986), it is unlikely that the control of development is due to a single limiting factor, rather, it is more probable that the rates of many inter-connected metabolic processes determine growth rate.

Grain removal experiments which follow a different pattern, for example removal of distal florets could be used to determine the full yield potential of A and B grains as compared to C and D grains. In addition the relationship

between whole spikelets, possibly in terms of hormonal influence (Singh and Jenner, 1982b, Evans, 1981), vascular supply (Cook and Evans, 1983; Hanif and Langer, 1972) or stage of development, and hence ability to compete, may be investigated by a varied pattern of spikelet removal or whole spikelet sterilisation.

Comparison of the modal volume of A type starch granules and the ratio of B types to A types in Chapter 3, with data for other wheat varieties (Chojecki et al., 1986b), revealed that these are cultivar specific traits. It was also considered that these parameters are not as affected by extrinsic factors as is endosperm cell number (see Table 3.4). The control of these characteristics is clearly a major determinant of grain starch content, therefore further investigation into both the biochemistry and genetics of these is likely to be of considerable agronomic importance.

An understanding of the control of the cessation of grain filling is also of primary importance in the determination of grain yield potential. It has been suggested that pericarp photosynthesis, and hence oxygen supply to the endosperm may have an effect (Duffus, 1979; Gifford and Bremner, 1981) and that this may be limited in some way by hormonal control (McWha, 1975; Radley, 1976;1978).

Cell and plastid division cycle

Results presented in chapter 3 revealed that wheat endosperm amyloplast number and volume does not appear to be

tightly coupled to nDNA content. This is in contrast to total chloroplast face area per cell which was found to be directly related to nuclear ploidy (Ellis and Leech, 1985).

Degraining resulted in a more rapid rate, but not duration, of endosperm cell division, but the rate of appearance of starch granules did not increase to the same degree. As a result starch granule and A type amyloplast number per cell were 19% and 18%, respectively, lower in the third grain of degrained spikes, as compared to intact spikes. From this it would appear that plastid division,or possibly starch granule initiation, although responsive to degraining, was limited relative to cell division and nDNA synthesis. This is in agreement with current data which indicates that cell division is reasonably responsive to factors external to the grain, whereas starch synthesis appears to be more intrinsically limited (Table 3.4 and Fig. 3.4).

Whether the cell storage protein reserves are similarly limited is not evident from this work. However, Radley and Thorne (1981) found that giant grains from degrained spikes contained a higher protein:starch ratio and it is known that protein deposition is less sensitive to high temperatures (Bhullar and Jenner, 1985; 1986) and other environmental stresses (Brooks et al., 1982; Jenner, 1979) than is the accumulation of starch. If it were possible that grain size could be increased genetically, as it is by degraining, it is of significance that this may also result in an improved protein yield.

Also important is the fact that the rate of plastid division may be unable to increase to the same degree as cell division. If this were to be true for other plants and tissues, as well as wheat endosperm, it is possible that plastid division may be a limiting factor in the yield of some crops.

The wheat endosperm amyloplast genome

In chapter 4 it was estimated that wheat endosperm cells contain from 3340 to 5570 plastome copies, whereas in $4\frac{1}{2}$ wk old wheat leaves the plastome copy number per cell was up to ten fold greater at 33500, as estimated in this thesis and from Boffey and Leech (1982). However, since the number of chloroplast plastomes per plastid (216) was estimated to be of a similar order of magnitude to plastome number per A type amyloplast (from 96 to 393 or less) it was suggested that the amyloplast plastome may be transcriptionally active. Yet, Southern blots presented in chapter 5 only detected total endosperm RNA transcripts with homology to the P6 and S8, rRNA bearing gene fragments of the chloroplast genome.

These results raise a number of questions which remain to be answered. It has not yet been conclusively determined that the amyloplast is not transcriptionally active, although the endosperm 16S rRNA appears to be expressed at a level equivalent to 18-51% relative to the leaf, or less (see also Macherel et al., 1986b; Piechulla et al., 1986). It would seem unlikely that the plastid genome would be replicated throughout endosperm growth, until the onset of maturation,

if it is never functionally active. However, it may be that the plastome is transcribed in the immature endosperm and the rRNA transcripts survive through to grain maturity because of their relative stability (Mullet and Klein, 1987).

Since proplastids or leucoplasts which either contain no 70S ribosomes (Carde, 1984; Steele Scott et al., 1982; Strzalka et al., 1984; Walbot and Coe, 1979; Höinghaus and Feierabend, 1985) or a severely reduced plastome (Day and Ellis, 1984, 1985; Ellis and Day, 1986) are known to be able to undergo both plastid division and genome replication it would appear that the genes for these functions are nuclear encoded. In fact it is not known whether the O.R.F.'s present in the plastid genome encode genes for plastid functions other than photosynthesis (Höinghaus and Feierabend, 1985); although results presented in chapter 5 revealed that at least 30% of the wheat plastome was not transcribed much, if at all, in chloroplasts from $4\frac{1}{2}$ week old leaves. It is extremely probable that these regions will include a number of O.R.F.'s (see Shinozaki et al., 1986; Ohyama et al., 1986) and it is conceivable that these may encode proteins with a role in translocator activities, plastid tubule membranes or possibly other functions specific to different plastid types.

It has also not been determined whether any of these O.R.F.'s may encode proteins involved in starch metabolism in the chloroplast, and/or amyloplast, or whether these genes are all nuclear encoded as has been determined so far (Boyer, 1985; Nishimura et al., 1987; Nolan et al., 1987; Chourey et

al., 1986; Kurzok and Feirabend, 1986). Of particular relevance to this is whether any of the ribosome less mutant plastids are able to synthesise starch, as indicated by Swanson et al. (1983), or whether they remain totally undifferentiated (Höinghaus and Feierabend, 1985; Steele Scott et al. 1982; or resemble leucoplasts (Carde, 1984). No plastid encoded genes have yet been detected which are light repressed (Link, 1984), but light independent in vitro transcription in chromophytic plastids of Olisthodiscus luteus has been reported (Reith and Cattolico, 1985). In addition, as discussed in chapter 5, etioplasts are known to be transcriptionally, and translationally, active and they show a different pattern to that found in chloroplasts (Miller et al., 1983).

Also of interest, with respect to a number of aspects of plant manipulation, are the enhancer and promoter sequences upstream of the DNA sequences coding for proteins imported into plastids other than chloroplasts and also the transit peptide sequences of the precursor proteins (Smeekens et al., 1986; Karlin-Neumann and Tobin, 1986). It has been shown that castor bean leucoplasts are able to import and process the precursor to the small subunit of ribulose bisphosphate carboxylase bearing the transit peptide which would normally locate the protein into the chloroplast stroma (Boyle et al., 1986). It is also of importance to determine whether RNA polymerase activity in the chloroplast can be attributed to a single enzyme, some of the subunits of which are known to be chloroplast encoded (Shinozaki et al., 1986; Ohyama et al.,

1986) or whether there may be a modifying component (Mullet and Klein, 1987) involved in transcription from different promoters, such as that for the 16S rRNA gene (see also Siemenroth et al., 1981).

This project was initiated with the initial intention of investigating the increase in wheat grain size resulting from grain removal experiments, with particular emphasis on starch granule numbers and sizes and cell number. Results presented here reveal that this increased capacity was attained, in the cultivar Timmo, primarily by more rapid cell division in the endosperm and to a lesser extent by an increase in the rate of starch granule initiation. The second aim was to determine whether endosperm amyloplasts contain ptDNA and, if so, whether this DNA is transcriptionally active. From this work it appears that A type amyloplasts contain ptDNA which shows homology to ctDNA and that the ribosomal genes of the amyloplast plastome appear to be transcribed during endosperm development. It yet remains to be determined whether this genome is necessary for the development of A type amyloplasts.

REFERENCES

Anderson, M.L.M. & Young, B.D. (1985) In Nucleic acid hybridisation a practical approach (eds. B.D. Hames & S.J. Higgins) pp. 73-112. IRL Press: Oxford.

Aguettaz, P., Seyer, P., Pesey, H. & Lescure, A.M. (1987). Plant Molecular Biology, 8, 169-177.

Asana, R.D., Parvatikar, S.R. & Saxena, N.P. (1969). Physiologia Plantarum, 22, 915-924.

Aspinall, D. (1984). In Control of crop productivity (ed. C.J. Pearson) pp. 91-110. Academic Press: Sydney.

Austin, R.B., Bingham, J., Blackwell, R.D., Evans, L.T., Ford, M.A., Morgan, C.L. & Taylor, M. (1980) Journal of Agricultural Science, 94, 675-689.

Baldo, B.A., Boniface, P.A. & Simmonds, D.H. (1982) Australia Journal of Plant Physiology, 9, 647-661.

Barlow, P.W. (1985) Annals of Botany, 55, 445-457.

Bartels, D. & Thompson, R.D. (1986) Plant Science, 46, 117-125.

Baruch, D.W., Meredith, P., Jenkins, L.D. & Simmons, L.D. (1979) Cereal Chemistry, 56, 554-558.

Baruch, D.W., Jenkins, L.D., Dengate, H.N. & Meredith, P. (1982) Cereal Chemistry., 60, 32-35.

Bechtel, D.B., Gaines, R.L. & Pomeranz, Y. (1982) Annals of Botany, 50, 507-518.

Bedbrook, J.R., Jones, J., O'Dell, M., Thompson, R.D. & Flavell, R.B. (1980) Cell, 19, 545-560.

Bedbrook, J.R. (1982) In Methods in Chloroplast Molecular

Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp. 521-530. Elsevier Biomedical Press: Amsterdam.

Bennett M.D. (1972) Proceedings of the Royal Society. London B, 181, 109-135.

Bennett, M.D., Rao, M.R., Smith, J.B. & Bayliss, M.W. (1973) Philosophical Transactions of the Royal Society, London B, 266, 39-81.

Bennett, M.D., Smith, J.B. & Barclay, I. (1975) Philosophical Transactions of the Royal Society. London B, 272, 199-227.

Bennett, M.D. & Smith, J.B. (1976) Philosophical Transactions of the Royal Society. London B, 274, 227-274.

Bhullar, S.S. & Jenner, C.F. (1985) Australian Journal of Plant Physiology, 12, 363-375.

Bhullar, S.S. & Jenner, C.F. (1986a) Australian Journal of Plant Physiology, 13, 605-615.

Bhullar, S.S. & Jenner, C.F. (1986b) Australian Journal of Plant Physiology, 13, 617-626.

Bingham, S.E. & Hallick, R.B. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp. 437-444. Elsevier Biomedical Press: Amsterdam.

Birnstiel, M.L., Sells, B.H. & Purdom, I.F. (1972) Journal of Molecular Biology, 63, 21-39.

Blin, N. & Stafford, D.W. (1976) Nucleic Acids Research, 3, 2303-2308.

Boasson, R. & Laetsch, W.M. (1969) Science, 166, 749-751.

Boffey, S.A. (1985) In The Cell Division Cycle in Plants

(eds. J.A. Bryant & D. Francis) pp. 233-246. Society for Experimental Biology. Seminar Series 26. Cambridge University Press: Cambridge.

Boffey, S.A., Ellis, J.R., Sellden, G. & Leech, R.M. (1979) Plant Physiology, 64, 502-505.

Boffey, S.A. & Leech, R.M. (1982) Plant Physiology, 69, 1387-1391.

Böhm, N. & Sprenger, E. (1968) Histochemie, 16, 100-118.

Bondar, P.I., Karimov, M., Lobov, V.P. & Vinetsky, Yu.P. (1979) Fiziologiya Biokhimiya, 11, 271-275.

Borroto, K.E. & Dure, L. (1986) Plant Molecular Biology, 7, 105-113.

Boutry, M. & Chua, N.-H. (1985) The EMBO Journal, 4, 2159-2165.

Bowman, C.M. (1986) Planta, 167, 264-274.

Bowman, C.M. & Dyer, T.A. (1982) Analytical Biochemistry, 122, 108-118.

Bowman, C.M., Koller, B., Delius, H. & Dyer, T.A. (1981) Molecular and General Genetics, 183, 93-101.

Boyer, H.W. & Roulland-Dussoix, D. (1969) Journal of Molecular Biology, 41, 459-472.

Boyer, C.D. (1985) In Biochemical basis of plant breeding. Vol. 1. Carbon metabolism (ed. C.A. Neyra) pp. 133-153. CRC Press: New York.

Boyle, S.A., Hemmingsen, S.M. & Dennis, D.T. (1986) Plant Physiology, 81, 817-822.

Bremner, P.M. (1972) Australian Journal of Biological Science, 25, 657-668.

Bremner, P.M. & Rawson, H.M. (1978) Australian Journal of Plant Physiology, 5, 61-72.

Briarty, L.G., Hughes, C.E. & Evers, A.D. (1979) Annals of Botany, 44, 641-658.

Britten, R.J. & Kohne, D.E. (1968) Science, 161, 529-540.

Britten, R.J., Graham, D.E. & Neufeld, B.R. (1974) Methods in Enzymology, 29, 363-406.

Brocklehurst, P.A. (1977) Nature, 266, 348-349.

Brocklehurst, P.A. & Evers, A.D. (1977) Journal of the Science of Food and Agriculture, 28, 1084-1089.

Brooks, A., Jenner C.F. & Aspinall, D. (1982) Australian Journal of Plant Physiology, 9, 423-436.

Burton, K. (1956) Biochemistry, 62, 315-323.

Butterfass, T. (1973) Protoplasma, 76, 167-195.

Butterfass, T. (1983) Protoplasma, 118, 71-74.

Buttrose, M.S. (1960) Journal of Ultrastructure Research, 4, 231-257.

Buttrose, M.S. (1963) Australian Journal of Biological Science, 16, 305-322.

Cannon, G., Heinhorst, S., Siedlecki, J. & Weissbach, A. (1985a) Plant Cell Reports, 4, 41-45.

Cannon, G., Heinhorst, S. & Weissbach, A. (1985b) Analytical Biochemistry, 149, 229-237.

Cannon, G.C., Heinhorst, S. & Weissbach, A. (1986) Plant Molecular Biology., 7, 331-341.

Carde, J.-P. (1984) European Journal of Cell Biology, 34, 18-26.

Carlberg, T., Glimelius, K., Eriksson, T. (1984) Plant

Science Letters, 35, 225-230.

Carmichael, G.G. & McMaster, G.K. (1980) Methods in Enzymology, 65, 380-391.

Cattolico, R.A. (1978) Plant Physiology, 62, 558-562.

Cavalier-Smith, T. (1978) Journal of Cell Science, 34, 247-278.

Chelm, B.K. & Hoben, P.J. & Hallick, R.B. (1977) Biochemistry, 16, 776-781.

Chelm, B.K. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp. 301-316.

Chojecki, A.J.S. (1985) Genetic and Developmental Studies of Grain Size in Wheat. D. Phil. Thesis: Cambridge.

Chojecki, A.J.S., Bayliss, M.W. & Gale, M.D. (1986a) Annals of Botany, 58, 809-818.

Chojecki, A.J.S., Gale, M.D. & Bayliss, M.W. (1986b) Annals of Botany, 58, 819-832.

Chourey, P.S. (1982) In Maize for Biological Research (ed. W.F. Sheridan) pp. 129-134. Plant Molecular Biology Association.

Chourey, P.S., Latham, M.D. & Still, P.E. (1986) Molecular and General Genetics, 203, 251-255.

Chua, N.-H. & Schmidt, G.W. (1979) Journal of Cell Biology, 81, 461-483.

Cochrane, M.P. (1983) Australian Journal of Plant Physiology, 10, 473-491.

Cochrane, M.P. & Duffus, C.M. (1983) Annals of Applied Biology, 102, 177-181.

Colman, A. & Robinson, C. (1986) Cell, 46, 321-322.

Conover, J.C. & Pryke, J.A. (1987) Journal of Experimental Botany, 38, 1219-1227.

Cook, M.G. & Evans, L.T. (1983) Australian Journal of Plant Physiology, 10, 313-327.

Cosgrove-Keown, A., Taiz, L. & Jones, R.L. (1977) American Journal of Botany, 64, 1248-1253.

Courtice, G.R.M., Bowman, C.M., Dyer, T.A. & Gray, J.C. (1985) Current Genetics, 10, 329-333.

Crossway, A. & Houck, C.M. (1985) Plant Molecular Biology, 5, 183-190.

Dagert, M. & Ehrlich, S.D. (1979) Gene, 6, 23-28.

Day, A. & Ellis, T.H.N. (1984) Cell, 39, 359-368.

Day, A. & Ellis, T.H.N. (1985) Current Genetics, 9, 671-678.

Davidson, J.C. & Birch, J.W. (1980) Proceedings of the Australian Agronomy Conference, Queensland Agricultural College, p.231.

Davies, D.R. (1976) Plant Science Letters, 7, 17-25.

Dawson, A.J., Hodge, T.P., Isaac, P.G., Leaver, C.J. & Lonsdale, D.M. (1986) Current Genetics, 10, 561-564.

Dean, C. & Leech, R.M. (1982a) Plant Physiology, 69, 904-910.

Dean, C. & Leech, R.M.(1982b) Plant Physiology, 70, 1605-1608.

Denhardt, D.T. (1966) Biochemistry and Biophysics Research Communications, 23, 641-646.

Donald, C.M. (1981) In Wheat Science - Today and Tomorrow (eds. L.T. Evans & W.J. Peacock) p.223. Cambridge University Press: Cambridge.

Donovan, G.R. (1979) Australian Journal of Plant Physiology, 6, 449-457.

Donovan, G.R. (1983) Australian Journal of Plant Physiology, 10, 569-572.

Donovan, G.R., Lee, J.W., Longhurst, T.J. & Martin, P. (1983a) Australian Journal of Plant Physiology, 10, 445-450.

Donovan, G.R., Jenner, C.F., Lee, J.W. & Martin, P. (1983b) Australian Journal of Plant Physiology, 10, 31-42.

Dunham, V.L. & Bryant, J.A. (1983) In Isolation of Membranes and Organelles from Plant Cells (eds. J.L. Hall & A.L. Moore) pp. 237-276. Academic Press: London.

Dunstone, R.L. & Evans, L.T. (1974) Australian Journal of Plant Physiology, 1, 157-165.

Duffus, C.M. (1979) In Recent Advances in the Biochemistry of Cereals (eds. D.L. Laidman & R.G. Wyn Jones) pp. 209-238. Academic Press: London.

Dyer, T.A. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp. 359-368. Elsevier Biomedical Press: Amsterdam.

Echeverria, E., Boyer, C., Liu, K.-C. & Shannon, J. (1985) Plant Physiology, 77, 513-519.

Ellis, R.J. (1979) Trends in Biochemical Science, 4(11), 241-244.

Ellis, J.R., Jellings, A.J. & Leech, R.M.(1983) Planta, 157, 376-380.

Ellis, J.R. & Leech, R.M.(1985) Planta, 165, 120-125.

Ellis, T.H.N. & Day, A. (1986) The EMBO Journal, 5(11),

2769-2774.

Emes, M.J. & England, S. (1986) Planta, 168, 161-166.

Ersland, D.R., Aldrich, J. & Cattolico, R.A. (1981) Plant Physiology, 68, 1468-1473.

Evans, L.T. (1978) Field Crops Research, 1, 5-19.

Evans, L.T. (1981) In Wheat Science - Today and Tomorrow (eds. L.T. Evans & W.J. Peacock) pp. 203-222. Cambridge University Press: Cambridge.

Evans, L.T. & Dunstone, R.L. (1970) Australian Journal of Biological Science, 23, 725-741.

Evans, L.T., Dunstone, R.L., Rawson, H.M. & Williams, R.F. (1970) Australian Journal of Biological Science, 23, 743-752.

Evans, L.T., Bingham, J. & Roskams, M.A. (1972) Australian Journal of Biological Science, 25, 1-8.

Evans, L.T. & Wardlaw, I.F. (1976) Advances in Agronomy, 28, 301-359.

Evers, A.D. (1970) Annals of Botany, 34, 547-555.

Evers, A.D. (1971) Die Stärke, 23, 157-192.

Evers, A.D. (1973) Die Stärke, 25, 303-304.

Evers, A.D. (1974) Proceedings of the 4th International Congress of Food Science and Technology, Madrid, 1, 422-431.

Evers, A.D. & Lindley, J. (1977) Journal of the Science of Food and Agriculture, 28, 98-102.

Felker, F.C., Peterson, D.M. & Nelson, O.E. (1983) Plant Physiology, 72, 679-684.

Fischer, R.A. & HilleRisLambers, D. (1978) Australian Journal

of Agricultural Research, 29, 443-458.

Fischer, R.A. & Stockman, Y.M. (1980) Australian Journal of Physiology, 7, 169-180.

Fluhr, R., Hassner, L., Edelman, M., Givol, D. & Mory, Y. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman et al.) pp. 507-521. Elsevier Biomedical Press: New York.

Galli, M.G., Balzaretti, R. & Sgorbati, S. (1986) Journal of Experimental Botany, 37, 1716-1724.

Gaynor, J.J. & Galston, A.W. (1983) Plant and Cell Physiology, 24, 411-421.

Gellissen, G., Bradfield, J.Y., White, B.N. & Wyatt, G.R. (1983) Nature, 301, 631-634.

Gerlach, W.L. & Bedbrook, J.R. (1979) Nucleic Acids Research, 7, 1869-1885.

Gifford, R.M. & Bremner, P.M. (1981) Australian Journal of Plant Physiology, 8, 631-640.

Gifford, R.M. & Evans, L.T. (1981) Annual Review of Plant Physiology, 32, 485-509.

Giles, K.W. & Myers, A. (1965) Nature, 206, 93.

Gillespie, D. (1968) Methods in Enzymology, 12B, 641-688.

Gleadow, R.M., Dalling, M.J. & Halloran, G.M. (1982) Australian Journal of Plant Physiology, 9, 539-551.

Gomori, G. (1955) Methods in Enzymology, 1, 138-146.

Greene, F.C. (1983) Plant Physiology, 71, 40-46.

Gross, P. & ap Rees, T. (1986) Planta, 167, 140-145.

Gruenbaum, Y., Cedar, H. & Razin, A. (1981a) Nucleic Acids Research, 9, 2509-2515.

Gruenbaum, Y., Naveh-Many, T., Cedar, H. & Razin, A. (1981b) Nature, 292, 860-862.

Guilfoyle, T.J., Suzich, J. & Lindberg, M. (1986) Plant Molecular Biology, 7, 95-104.

Hageman, J., Robinson, C., Smeekens, S. & Weisbeek, P. (1986) Nature, 324, 567-569.

Hallick, R.B., Rushlow, K.E. & Bingham, S.E. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman R.B. Hallick & N.-H. Chua) pp. 315-337. Elsevier Biomedical Press: Amsterdam.

Hansmann, P., Falk, H., Ronai, K. & Sitte, P. (1985) Planta, 164, 459-472.

Harper, J.L., Lovell, P.H. & Moore, K.G. (1970) Annual Review of Ecology and Systematics, 1, 327-356.

Hanif, M. & Langer, R.H.M. (1972) Annals of Botany, 36, 721-727.

Heidecker, G. & Messing, J. (1986) Annual Review of Plant Physiology, 37, 439-466.

Heinhorst, S., Cannon, G. & Weissbach, A. (1985) Plant Molecular Biology, 4, 3-12.

Hendrix, J.E., Linden, J.C., Smith, D.H., Ross, C.W. & Park, I.K. (1986) Australian Journal of Plant Physiology, 13, 391-398.

Herrmann, R.G. (1972) Protoplasma, 74, 7-17.

Herrmann, R.G. & Possingham, J.V. (1980) In Chloroplasts (ed. J. Reinert) pp. 45-96. Springer-Verlag: Berlin.

Herrmann, R.G. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp.

259-284. Elsevier Biomedical Press: Amsterdam.

Herzog, H. & Stamp, P. (1983) Euphytica, 32, 511-520.

Hird, S.M., Willey, D.L., Dyer, T.A. & Gray, J.C. (1986a) Molecular and General Genetics, 203, 95-100.

Hird, S.M., Dyer, T.A. & Gray, J.C. (1986b) FEBS Letters, 209, 181-186.

Höinghaus, R. & Feierabend, J. (1985) Planta, 166, 452-465.

Honda, S.I., Hongladoram, T., Laties, G.G. (1966) Journal of Experimental Botany, 17, 460-472.

Hughes, C.E. & Briarty, L.G. (1976) Die Stärke, 28, 336-337.

Hussein, Y., Heizmann, P., Nicolas, P. & Nigon, V. (1982) Current Genetics, 6, 111-117.

Isaac, P.G., Brennicke, A., Dunbar, S.M. & Leaver, C.J. (1985) Current Genetics, 10, 321-328.

Iwatsuki, N., Hirai, A. & Asahi, T. (1985) Plant Cell Physiology, 26, 599-601.

Jacobsen, E., Tempelaar, M.J. & Bijmolt, E.W. (1983) Theoretical and Applied Genetics, 65, 113-118.

James, T.W. & Jope, C. (1978) Journal of Cell Biology, 79, 623-630.

Jellings, A.J. & Leech, R.M. (1984) New Phytologist, 96, 371-382.

Jenkins, G.I., Hartley, M.R. & Bennett, J. (1983) Philosophical Transactions of the Royal Society London B, 303, 419-431.

Jenner, C.F. (1973) Journal of Experimental Botany, 24, 295-306.

Jenner, C.F. (1974) In Mechanisms of Regulation of Plant

Growth (eds. R.L. Beileski, A.R. Ferguson & M.N.R. Cresswell) pp. 901-908. Royal Society of New Zealand Bulletin, 12.

Jenner, C.F. (1976) Australian Journal of Plant Physiology, 3, 337-347.

Jenner, C.F. (1979) Australian Journal of Plant Physiology, 6, 629-641.

Jenner, C.F. (1980) Australian Journal of Plant Physiology, 7, 113-121.

Jenner, C.F. (1982a) In Encyclopedia of Plant Physiology Plant Carbohydrates N.S. 13a (eds. P.A. Loewus & W. Tanner) pp. 700-747. Springer Verlag: Berlin.

Jenner, C.F. (1982b) Australian Journal of Plant Physiology, 9, 69-82.

Jenner, C.F. (1985a) Australian Journal of Plant Physiology, 12, 573-586.

Jenner, C.F. (1985b) Australian Journal of Plant Physiology, 12, 587-594.

Jenner, C.F. (1985c) Australian Journal of Plant Physiology, 12, 595-607.

Jenner, C.F. & Rathjen, A.J. (1972a) Annals of Botany, 36, 729-741.

Jenner, C.F. & Rathjen, A.J. (1972b) Annals of Botany, 36, 743-754.

Jenner, C.F. & Rathjen, A.J. (1975) Australian Journal of Plant Physiology, 2, 311-322.

Jenner, C.F. & Rathjen, A.J. (1977) Australian Journal of Plant Physiology, 4, 691-701.

Jenner, C.F. & Rathjen, A.J. (1978) Australian Journal of Plant Physiology, 5, 249-262.

Jones, M.C. & Boffey, S.A. (1984) FEBS Letters, 174, 215-218.

Kafatos, F.C., Jones, W.C. & Efstratiadis, A. (1978) Nucleic Acids Research, 7, 1541-1552.

Kapuscinski, J. & Szer, W. (1979) Nucleic Acids Research, 6, 3519-3534.

Karimov, M., Lysenko, A.M. & Vinetskii, Y.P. (1978) Biokhimiya, 43, 597-601.

Karlin-Neumann, G.A. & Tobin, E.M. (1986) The EMBO Journal, 5, 9-13.

Kellogg, T.F. (1983) Analytical Biochemistry, 134, 137-143.

Kessler, C. & Höltke, H.-J. (1986) Gene, 47, 1-153.

Kirk, J.T.O. & Tilney-Bassett, R.A.E. (1978) The Plastids. Elsevier Biomedical Press: Amsterdam.

Kislev, N. & Rubenstein, I. (1980) Plant Physiology, 66, 1140-1143.

Koller, B., Delius, H. & Dyer, T.A. (1982) European Journal of Biochemistry, 122, 17-23.

Kolodner, R. & Tewari, K.K. (1975) Biochimica et Biophysica Acta, 402, 372-390.

Kreuz, K., Dehesh, K. & Apel, K. (1986) European Journal of Biochemistry, 159, 459-467.

Kumar, R. & Singh, R. (1980) Phytochemistry, 19, 2299-2303.

Kuroiwa, T. & Suzuki, T. (1981) Experimental Cell Research, 134, 457-460.

Kuroiwa, T., Suzuki, T., Ogawa, K. & Kawano, S. (1981) Plant and Cell Physiology, 22, 381-398.

Kurzok, H.-G. & Feierabend, J. (1986) Theoretical and Applied Genetics, 72, 359-363.

Lamppa, G.K. & Bendich, A.J. (1979a) Plant Physiology, 64, 126-130.

Lamppa, G.K. & Bendich, A.J. (1979b) Plant Physiology, 63, 660-668.

Lamppa, G.K., Elliot, L.V. & Bendich, A.J. (1980) Planta, 148, 437-443.

Lamppa, G.K. & Bendich, A.J. (1984) Planta, 162, 463-468.

Laskey, R.A. & Mills, A.D. (1975) European Journal of Biochemistry, 56, 335-341.

Laskey, R.A. (1980) Methods in Enzymology, 65, 363-371.

Leegood, R.C. & Walker, D.A. (1983) In Isolation of Membranes and Organelles from Plant Cells (eds. J.L. Hall & A.L. Moore) pp. 185-210. Academic Press: London.

Leech, R.M., Leese, B.M. & Jellings, A.J. (1985) Planta, 166, 259-263.

Lin, B.-Y. (1977) The Journal of Heredity, 68, 143-149.

Liu, T-T.Y. & Shannon, J.C. (1981) Plant Physiology, 67, 518-524.

Link, G. (1984) Plant Molecular Biology, 3, 243-248.

Lobov, V.P. & Bondar, P.I. (1977) Fiziologiya Rastenii, 24, 318-322.

Lobov, V.P. & Petrov, I.A. (1983) Soviet Plant Physiology, 30(6), 824-833.

Loening, U.E. (1967) Biochemical Journal, 102, 251-257.

Lonsdale, D.M., Hodge, T.P., Howe, C.J. & Stern, D.B. (1983) Cell, 34, 1007-1014.

Luthe, D.S. & Quatrano, R.S. (1980) Plant Physiology, 65, 305-308.

MacDonald, F.D. & ap Rees, T. (1983) Biochimica et Biophysica Acta, 755, 81-89.

MacDonald, F.D. & Preiss, J. (1986) Planta, 167, 240-245.

MacDowell Dale, E. & Housley, T.L. (1986) Plant Physiology, 82, 7-10.

Macherel, D., Kobayashi, H. & Akazawa, T. (1985) Biochemical Biophysical Research Communication, 133, 140-146.

Macherel, D., Viale, A. & Akazawa, T. (1986a) Plant Physiology, 80, 1041-1044.

Macherel, D., Kobayashi, H., Valle, E. & Akazawa, T. (1986b) FEBS Letters, 201, 315-320.

Maherchandani, N.J. & Naylor, J.M. (1971) Canadian Journal of Genetics and Cytology, 13, 578-584.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Press: New York.

Maxwell, I.H., Van Ness, J. & Hahn, W.E. (1978) Nucleic Acids Research, 5, 2033-2038.

May, L.H. & Buttrose, M.S. (1959) Australian Journal of Plant Physiology, 12, 146-159.

Migniniac-Maslow, M., Hoaran, A. & Moyse, A. (1979) Zeitschrift für Pflanzenphysiologie, 95, 95-104.

Miller, M.E., Jurgenson, J.E., Reardon, E.M. & Price, C.A. (1983) Journal of Biological Chemistry, 358, 4478-4484.

Millerd, A. & Whitfield, P.R. (1973) Plant Physiology, 51, 1005-1010.

Mohapatra, P.K., Aspinall, D. & Jenner, C.F. (1983a) Australian Journal of Plant Physiology, 10, 1-7.

Mohapatra, P.K., Aspinall, D. & Jenner, C.F. (1983b) Australian Journal of Plant Physiology, 10, 493-502.

Morgan, J.M. & King, R.W. (1984) Australian Journal of Plant Physiology, 11, 143-150.

Morrison, I.N. & O'Brien, T.P. (1976) Planta, 130, 57-67.

Morrison, I.N., Kuo, J. & O'Brien, T.P. (1975) Planta, 123, 105-116.

Morrison, I.N., O'Brien, T.P. & Kuo, J. (1978) Planta, 140, 19-30.

Mubumbila, M., Bowman, C.M., Droog, F., Dyer, T., Kuntz, M. & Weil, J.H. (1985) Plant Molecular Biology, 4, 315-320.

Mullett, J.E. & Klein, R.R. (1987) The EMBO Journal, 6, 1571-1579.

Munns, R., Brady, C.J. & Barlow, E.W.R. (1979) Australian Journal of Plant Physiology, 6, 379-389.

Müntz, K. (1982) In Cell Differentiation Molecular Basis and Problems (eds. L. Nover, M. Luckner & Parthier, B.) pp. 427-448. Springer-Verlag: Berlin.

McMaster, G.K. & Carmichael, G.G. (1977) Biochemistry, 74, 4835-4838.

McLeish, J. & Sunderland, N. (1961) Experimental Cell Research, 24, 527-540.

McWha, J.A. (1975) Journal of Experimental Botany, 26, 823-827.

Nagashima, H., Kuroiwa, T. & Fukuda, I. (1984) Experientia, 40, 563-564.

Nagl, W. (1978) International Review of Cytology, 94, 21-57.

Nagl, W. Pohl, J. & Radler, A. (1985) In The cell division cycle in plants (eds. J.A. Bryant & D. Francis) pp. 217-232. Cambridge University Pres: Cambridge.

Nicolas, M.E., Gleadow, R.M. & Dalling, M.J. (1984) Australian Journal of Plant Physiology, 11, 553-566.

Nicolas, M.E., Gleadow, R.M. & Dalling, M.J. (1985) Annals of Botany, 55, 433-444.

Nishimura, M., Hara-Nishimura, I,. Bureau, D. & Daussant, J. (1987) Plant Science, 49, 117-122.

Nolan, R.C., Lin, L.-S. & Ho, T-h.D. (1987) Plant Molecular Biology, 8, 13-22.

Nösberger, J. & Thorne, G.N. (1965) Annals of Botany, 29, 635-644.

Obokata, J. (1984) Plant and Cell Physiology, 25, 821-830.

Ohyama, K, Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S-i., Inokuchi, H. & Ozeki, H. (1986) Nature, 322, 572-574.

Olszewska, M.J., Damsz, B. & Rabeda, E. (1983) Protoplasma, 116, 41-50.

Parker, M.L. (1985) Journal of Cereal Science, 3, 271-278.

Parthier, B. (1982) Biochemie und Physiologie der Pflanzen, 177, 283-317.

Palmer, J.D. (1985) Annual Review of Genetics, 19, 325-354.

Pernollet, J-C. & Vaillant, V. (1984) Plant Physiology, 76, 187-190.

Perrin, D., Langley, O.K. & Aunis, A. (1987) Nature,

326(6112), 498-501.

Piechulla, B., Pichevsky, E., Cashmore, A.R. & Gruissem, W. (1986) Plant Molecular Biology, 7, 367-376.

Pinthus, M.J. & Millet, E. (1978) Annals of Botany, 42, 839-848.

Pinthus, M.J. & Nerson, H. (1984) Australian Journal of Plant Physiology, 11, 17-22.

Pring, D.R. & Lonsdale, D.M. (1985) International Review of Cytology, 97, 1-46.

Pyke, K.A. & Leech, R.M. (1987) Planta, 170, 416-420.

Quigley, F., Grienenberger, J.M. & Weil, J.H. (1985) Plant Molecular Biology, 4, 305-310.

Quigley, F. & Weil, J.H. (1985) Current Genetics, 9, 495-503.

Radley, M. (1976) Journal of Experimental Botany, 27, 1009-1021.

Radley, M. (1978) Journal of Experimental Botany, 29, 919-934.

Radley, M. (1981) Journal of Experimental Botany, 32, 129-140.

Radley, M.E. & Thorne, G.N. (1981) Annals of Applied Biology, 98, 149-156.

Rawson, H.M. (1970) Australian Journal of Biological Science, 23, 1-15.

Rawson, H.M. & Evans, L.T. (1970) Australian Journal of Biological Science, 23, 753-764.

Rawson, J.R.Y. & Andrews, W.H. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp. 493-506. Elsevier Biomedical

Press: Amsterdam.

Reisfeld, A., Gressel, J., Jakob, K.M. & Edelman, M. (1982) In Methods in Chloroplast Molecular Biology (eds. M. Edelman, R.B. Hallick & N.-H. Chua) pp. 393-403. Elsevier Biomedical Press: Amsterdam.

Reith, M.E. & Cattolico, R.A. (1985) Biochemistry 24 (10), 2550-2556.

Richter, G. (1984) Plant Molecular Biology, 3, 271-276.

Rigby, P.W.J., Dieckman, M., Rhodes, C. & Berg, P. (1977) Journal of Molecular Biology, 113(1), 237-251.

Riley, R. (1981) In Wheat Science - Today and Tomorrow (eds. L.T. Evans & W.J. Peacock) pp. 271-277. Cambridge University Press: Cambridge.

Rijven, A.H.G.C. & Wardlaw, I.F., (1965) Experimental Cell Research, 41, 324-328.

Rijven, A.H.G.C. (1986) Plant Physiology, 81, 448-453.

Robinson, C. & Ellis, R.J. (1985) European Journal of Biochemistry, 152, 67-73.

Rocha, V. & Ting, I.P. (1970) Archives of Biochemistry and Biophysics, 140, 398-407.

Rochaix, J.D. (1984) International Review of Cytology, 93, 57-91.

Rodermel, S.R. & Bogorad, L. (1985) Journal of Cell Biology, 100, 463-476.

Rose, R.J., Cran, D.G. & Possingham, J.V. (1975) Journal of Cell Science, 17, 27-41.

Ruch, F. & Rosselet, A. (1970) Experimental Cell Research, 62, 219-227.

Salerno, G.L. (1986) Plant Science, 44, 111-117.

Saini, H.S. & Aspinall, D. (1982) Australian Journal of Plant Physiology, 1, 529-537.

Saini, H.S., Sedgley, M. & Aspinall, D. (1983) Australian Journal of Plant Physiology, 10, 137-144.

Sandstedt, R.M. (1946) Cereal Chemistry, 23, 337-359.

Sealey, P.G. & Southern, E.M. (1983) In Gel electrophoresis of nucleic acids; a practical approach (eds. D. Rickwood & B.D. Hames) pp. 39-75. Practical Approaches in Biochemistry Series. IRL Press.

Sellden, G. & Leech, R.M. (1981) Plant Physiology, 68, 731-734.

Seyer, P. & Lescure, A.M. (1984) Plant Science Letters, 36, 59-66.

Shannon, J.C. (1974) Cereal Chemistry, 51, 798-809.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H. & Sugiura, M. (1986) The EMBO Journal, 5, 2043-2049.

Siddell, S.G. & Ellis, R.J. (1975) Biochemical Journal, 146, 675-685.

Siemenroth, A., Wollgiehn, R., Neumann, D. & Börner, T. (1981) Planta, 153, 547-555.

Simmonds, D.H. & O'Brien, T.P. (1981) Advances in Cereal Science and Technology IV pp. 5-69. American Association

of Cereal Chemistry, St. Paul, MN.

Singh, B.K. & Jenner, C.F. (1982a) Plant Science Letters, 26, 273-278.

Singh, B.K. & Jenner, C.F. (1982b) Australian Journal of Plant Physiology, 9, 83-95.

Singh, B.K. & Jenner, C.F. (1984) Australian Journal of Plant Physiology, 11, 151-163.

Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. & Weisbeek, P. (1986) Cell, 46, 365-375.

Smillie, R.M. & Krotkov, G. (1960) Canadian Journal of Botany, 38, 31-49.

Smith, S.M. & Ellis, R.J. (1979) Nature, 278, 662-664.

Sofield, I., Evans, L.T., Cook, M.G. & Wardlaw, I.F. (1977a) Australian Journal of Plant Physiology, 4, 785-797.

Sofield, I., Evans, L.T., Cook, M.G. & Wardlaw, I.F. (1977b) Australian Journal of Plant Physiology, 4, 798-810.

Southern, E.M. (1975) Journal of Molecular Biology, 98, 503-517.

Springer, B., Werr, W., Starlinger, P., Bennett, D.C., Zkolica, M. & Freeling, M. (1986) Molecular and General Genetics, 205, 461-468.

Steele Scott, N. & Possingham, J.V. (1980) Journal of Experimental Botany, 31, 1081-1092.

Steele Scott, N., Cain, P. & Possingham, J.V. (1982) Zeitschrift für Pflanzenphysiologie, 108, 187-191.

Steele Scott, N. & Possingham, J.V. (1983) Journal of Experimental Botany, 34, 1756-1767.

Steele Scott, N., Tymms, M.J. & Possingham, J.V. (1984)

Planta, 161, 12-19.

Steele Scott, N. & Timmis, J.N. (1984) Theoretical and Applied Genetics, 67, 279-288.

Stern, D.B. & Lonsdale, D.M. (1982) Nature, 299, 698-702.

Stern, D.B. & Palmer, J.D. (1983) Carnegie Institute Washington. Annual Report Department of Plant Biology pp. 17-19.

Stockman, Y.M., Fischer, R.A. & Brittain, E.G. (1983) Australian Journal of Plant Physiology, 10, 585-594.

Stowell, R. (1945) Stain Technology, 20, 45-58.

Strzalka, K., Kwiatkowska, M. & Börner, T. (1984) Photosynthetica, 18(2), 266-269.

Sundqvist, C., Björn, L.O. & Virgin, I.L.L. (1980) In Chloroplasts (ed. J. Reinert) pp. 45-96. Springer-Verlag: Berlin.

Swanson, E.B., Tomes, D.T. & Hopkins, W.G. (1983) Canadian Journal of Botany, 61, 2500-2505.

Thomas, P.S. (1983) Methods in Enzymology, 100, 5201-5212.

Thomas, S.M., Thorne, G.N., Kendall, A.C. & Pearman, I. (1979) In Crop Physiology and Cereal Breeding (eds. J.H.J. Spiertz & T.H. Kramer) pp. 96-101. PUDOC: Wageningen.

Thorne, G.N. (1973) Rothamsted Report. Part 2, pp. 5-25.

Timmis, J.N. & Steele Scott, N. (1983) Nature, 305, 65-67.

Trewavas, A. (1986) Australian Journal of Plant Physiology,, 13, 447-457.

Tymms, M.J., Steele Scott, N. & Possingham, J.V. (1982) Journal of Experimental Botany, 33, 831-837.

Tymms, M.J., Steele Scott, N. & Possingham, J.V. (1983) Plant Physiology, 71, 785-788.

van den Boogaart, P., Samallo, J. & Agsteribbe, E. (1982) Nature, 298, 187-188.

Van Oostveldt, P. & Van Parijs, R. (1976) Experimental Cell Research, 98, 210-221.

Vedel, F., Quetier, F. & Bayen, M. (1976) Nature, 263, 401-402.

Vedel, F., Quetier, F., Dosba, F. & Doussinault, G. (1978) Plant Science Letters, 13, 97-102.

Verma, S.C. & Rees, H. Heredity, 33, 61-68.

Vytasek, R. (1982) Analytical Biochemistry, 120, 243-248.

Walbot, V. and Coe, E.H. (1979) Proceedings of the National Academy of Science. U.S.A., 76, 2760-2764.

Walbot, V., Thompson, D., Veith, G.M. & Coe, E.H. Jr. (1980) NATO Advanced Study Institute Series A: Life Sciences, 29, 381-399.

Warburg, O. and Christian, (1942) Biochemische Zeitung, 310, 384.

Wardlaw, I.F. (1970) Australian Journal of Biological Science, 23, 765-774.

Wardlaw, I.F. & Moncur, L. (1976) Planta, 128, 93-100.

Wardlaw, I.F., Sofield, I. & Cartwright, P.M. (1980) Australian Journal of Plant Physiology, 7, 387-400.

Warrington, I.J., Dunstone, R.L., Green, L.M. (1977) Australian Journal of Agricultural Research, 28, 11-27.

Waters, S.P., Martin, P. & Lee, B.T. (1984) Journal of Experimental Botany, 35, 829-840.

Whisson, D.L. & Steele Scott, N. (1985) Plant Molecular Biology, 4, 267-273.

White, B.A. & Bancroft, F.C. (1982) Journal of Biological Chemistry, 257, 8569-8572.

Whiteway, M.S. & Lee, R.W. (1977) Molecular and General Genetics, 157, 11-15.

Williams, J.M. & Duffus, C.M. (1977) Journal of the Institute of Brewing, 84, 47-50.

Wong, J.H.H. & Benedict, C.R. (1980)

Yang, R.C.-A., Lis, J. & Wu, R. (1979) Methods in Enzymology, 68, 176-182.

Yeoman, M.M. (1981) In The Cell Cycle (ed. P.C.L. John) pp. 181-184. Cambridge University Press: Cambridge.

Young, B.D. & Anderson, M.L.M. (1985) In Nucleic Acid Hybridisation a practical approach (eds. B.D. Hames & S.J. Higgins) pp. 47-72. IRL Press: Oxford.

Zee, S.Y. & O'Brien, T.P. (1970) Australian Journal of Biological Science, 23, 783-791.

Zee, S.Y. & O'Brien, T.P. (1971) Australian Journal of Biological Science, 24, 35-49.

Zelenskii, M.I., Mogileva, G., Shitora, I. & Fattakhova, F. (1978) Photosynthetica, 12, 428-435.

Zeng, Z.R., Morgan, J.M. & King, R.W. (1985) Australian Journal of Plant Physiology, 12, 609-619.

Zeng, Z.R. & King, R.W. (1986) Australian Journal of Plant Physiology, 13, 347-352.

Zuurendonk, P.F., Tischler, M.E., Akerboom, T.P.M., van der Meer, R., Williamson, J.R. & Tager, J.M. (1979) Methods in Enzymology, 56, 207-223.

APPENDIX I

Determination of optimum hydrolysis time for Feulgen stained wheat endosperm nuclei for fluorometric ploidy measurements

---

Introduction

Because fixative, hydrolysis time and stain all affect the reliability of Feulgen cytofluorometry, it was important that these should be optimised and kept contant (McLeish and Sunderland, 1961). The purpose of the experiments described here was to optimise the hydrolysis conditions to yield highly stained, intact endosperm nuclei, with low background fluorescence from the cytoplasm. Reproducibility and an even degree of staining across tissue sections are also important.

Materials and Method and Results

Grains weighing 20.0-22.5 mg were stored in acetic acid: ethanol and rehydrated prior to dissection (section 2.2.4). Endosperms were hydrolysed in either (i) 1 ml of 4M HCl at room temperature for 30 min and then at 40°C for 0, 10, 15, 20 or 30 min (Chojecki et al., 1986) or (ii) 1 ml of 1M HCl at 60°C for 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0 or 22.5 min (McLeish and Sunderland, 1961). Samples were then stained in Feulgen reagent as described in section 2.2.8.

Treatment	Duration of Incubation (min)	Comments on tissue staining and hydrolysis
(i) 4N HCl at room temperature (30 min) and at 40°C	0	Cells and starch granules largely intact but nuclei disrupted into cytoplasm. High background fluorescence
	10 and 15	Many nuclei and cells not intact. No starch granules. Uneven stain distribution. Some nuclei no longer visible.
	30	Cells and nuclei totally disrupted.
(ii) 1N HCl	20 and 22.5	Cells and nuclei totally disrupted. Starch granules not fluorescent.
	17	Nuclei more intact but irregular and fluorescence poor. Starch granules not fluorescent.
	15	Nuclei still more intact but background fluorescence relative to nucleus was high, possibly due to loss of nuclear DNA.
	12.5 and 10	Some aleurone cells intact but background fluorescence frequently greater than nuclear.
	10	Some free nuclei appeared intact
	7.5	Nuclei intact and heterogeneity of nuclear stain visible, fluorescence greater than that of cytoplasm

Treatment	Duration of Incubation (min)	Comments on tissue staining and hydrolysis
(i) 1N HCl (cont'd.)		
	5 and 2.5	Both starch granule-containing cells and nuclei were frequently intact. Starch granules not fluorescent and cytoplasm not much fluorescence
	2.5	Stain possibly less evenly distributed through tissue.

Discussion

It was determined that incubating endosperm slices in 4N HCl at room temperature for 30 min resulted in loss of starch granules from the cytoplasm and severe loss of nucleic acid from the nuclei into the cytoplasm. For endosperms treated with 1N HCl at 60°C for 2.5 to 7.5 min hydrolysis was less severe resulting in more even distribution of stain across the tissue. However with increasing incubation periods the nuclei became increasingly disperse, and stained more poorly, with a concomitant increase in cytoplasmic fluorescence. It was therefore decided that for intact, highly stained nuclei with low background fluorescence the acid treatment should be rapid, using 1N HCl at 60°C: an incubation period of 6 min was chosen for endosperm tissue. Stronger acid concentrations and long incubation periods should be avoided.

APPENDIX II

Optimisation of the S_1 nuclease assay to determine the percentage double stranded DNA

Introduction

Results of initial S_1 nuclease digestions indicated that the enzyme contained double stranded, as well as single stranded, nuclease activity. In addition, it was not clear whether the S_1 nuclease reaction and DNA binding of the DE81 filters were adversely affected by the presence of reassociation buffer; therefore, experiments were carried out to determine the optimal assay conditions.

Material and Methods and Results

Buffers and enzyme were as described in section 4.2.9.

(i) Effect of changing the ratio of reassociation buffer: S_1 nuclease buffer on filter binding of double stranded (dsDNA).

Samples were not digested with S_1 nuclease but were spotted directly onto DE81 filters, 50 μl per filter, and washed as described previously.

Table (i) Reassociation buffer: S_1 nuclease buffer ratio on dsDNA filter binding

Volume of reassociation buffer containing ds [^{3}H]ctDNA	Volume of S_1 nuclease buffer	Filters washed in phosphate buffer	C.p.m. per 10 µl of reassociation
20	90	✓	357
20	200	✓	408
10	210	✓	462
20	200	x	473

Results are means of duplicate samples.

From these results it appeared that for almost maximal DE81 binding of dsDNA the reassociation buffer, which contains 0.1% Sarcosyl, had to be diluted approximately 20 fold in S_1 nuclease buffer. These results were confirmed by a later assay containing enzyme.

(ii) Optimisation of the S_1 nuclease digestion

Carrier calf thymus DNA was preincubated at 100°C for 10 min and was diluted to 25 μg/ml in reassociation buffer. Reassociation buffer containing [^{3}H]ctDNA and single stranded (ss) calf thymus DNA was incubated at 60°C for 20 min, then half was incubated at 100°C for 10 min, and 20 μl aliquots were diluted in 400 μl of S_1 nuclease buffer. Enzyme was added at increasing concentrations to both ds and ss samples which were incubated at 37°C for either 2 or 4 h and then 200 μl was spotted onto each DE81 filter, as described in section 4.2.9.

Results revealed that ds [^{3}H]ctDNA which contained no carrier was 30.4% and 35.8% more digested by 10 units/420 μl of S_1 nuclease, for 2 and 4 h incubations, respectively, than were the equivalent samples containing carrier DNA (1 μg/ 420 μl). Thus for reassociations containing a standard DNA concentration of at least 25 μg/500 μl the assay is more optimal. For effective digestion of ssDNA with minimal digestion of dsDNA the S_1 nuclease digests should contain 5 units/420 μl, containing 1 μg of DNA, and should proceed for 2 h.

Table (ii) Optimisation of S_1 nuclease digestion

Treatment	Units of S_1 nuclease per 420 µl	C.p.m. per 200 µl of S_1 nuclease digestion			
		Duration of incubation 2 h		4 h	
		dsDNA	ssDNA	dsDNA	ssDNA
Control (not incubated)	0	352	293	390	285
Incubated	0	374	234	378	241
	1	303	31	307	23
	5	301	7	277	23
	10	263	24	240	23
	50	144	14	64	3
Control without carrier	10	183	12	154	31

Results are means of duplicate samples.

(iii) Effect of DNA and enzyme concentration on S_1 nuclease assay

Both the S_1 nuclease and the DNA concentrations in the S_1 nuclease assay were varied to determine the optima. [^{3}H]ctDNA was diluted in reassociation buffer with increasing amounts of ss calf thymus DNA. Each of the four carrier mixtures was diluted, 20 μl/420 μl, in S_1 nuclease buffer and incubated with three different enzyme concentrations for 2 h at 37°C.

Optimal results were obtained using between 1.2 to 3.6 μg of DNA per 420 μl of S_1 nuclease digestion, containing 5 units of enzyme, and incubated for 2 h at 37°C. This is equivalent to 30 to 90 μg per 500 μl of reassociation. Higher enzyme concentrations resulted in increased digestion of dsDNA and higher carrier concentrations may have resulted in loss of filter binding efficiency.

Discussion

A number of experiments were carried out using S_1 nuclease (from Aspergillus oryzae, Sigma) from different batches, and to varying extents all contained double stranded nuclease activity. For this reason it was important that the optimum enzyme to DNA ratio (of 5:1 to 5:4 here) and duration for the digestion (2 h) should be determined. These were related to the maximal DNA concentration in the S_1 nuclease digestion (3.6 μg/420 μl) for efficient binding to Whatman DE81 filters and to the optimal ratio of reassociation buffer: S_1 nuclease buffer (1:22) for almost 100% binding

Table (iii) Effect of DNA and enzyme concentration on S_1 nuclease assay

Carrier DNA in μg per 420 μl	Units of S_1 nuclease per 420 μl	C.p.m. per 200 μl of S_1 nuclease digestion	
		dsDNA	ssDNA
1.2	5	370	38
	15	292	-
	50	184	19
3.6	5	383	32
	15	315	23
	50	210	19
6.0	5	358	103
	15	296	20
	50	192	19

Results are means of duplicate samples.

efficiency. On the basis of these results it was considered that reassociation reactions should contain between 30 to 100 μg of DNA/500 μl.

APPENDIX III

Optimisation of the DE81 filter and TCA precipitation assays for double stranded DNA

Introduction

The previous experiments (Appendix II) defined the optimal S_1 nuclease assay conditions, including the ratio of reassociation buffer: S_1 nuclease for maximal DE81 filter binding of dsDNA. The following experiments were undertaken to determine the optimum conditions for DE81 filter washes and also for TCA precipitation of dsDNA onto glass fibre filters.

Materials and Methods and Results

Reagents and buffers were as described in section 4.2.9.

(i) Effects of increasing wash duration on DE81 binding to sonicated dsDNA.

[^{3}H]ctDNA was diluted in reassociation buffer, sonicated for 4 and 8 min as described in section 4.3.3, and one third was made single stranded by incubating at 100°C for 10 min. The S_1 nuclease assay was carried out as before (section 4.2.9) and 50 μl samples were spotted onto DE81 filters which were washed in 3 x 200 ml of phosphate buffer for a total of either 5 or 10 min.

Table (i) Wash duration for DE81 filters spotted with sonicated dsDNA

[^{3}H]ctDNA sonication treatment	Total wash duration (min)	C.p.m. per 50 µl of S_1 nuclease digestion	
		dsDNA	ssDNA
Control (not sonicated)	5	1158	142
	10	1070	145
4 min	5	1011	87
	10	972	68
8 min	5	810	59
	10	814	46

Results are means of duplicate samples.

Both sonicated DNA samples were of less than 400bp (section 4.3.3). It was evident from the results in Table (i) that sonication for 4 min or more did decrease binding efficiency compared with the nick translated control. DNA used for reassociations was only sonicated for 2.5 min and was therefore expected to bind almost as efficiently as non-sonicated.

It was found that washing filters for 10 min, rather than 5, resulted in a slight reduction in the efficiency of detection of dsDNA. An earlier experiment had indicated that there was no significant difference between filters washed for 1.5 and 3 min.

(ii) Comparison of the DE81 filter and TCA precipitation procedures for assay of dsDNA

S_1 nuclease digestion was carried out as suggested by the results of Appendix II. DE81 filters were spotted with 100 μl of digest and washed for a total of 5 min, as described in (i). For TCA precipitation, 100 μl of digest were mixed with 2 ml of 20% TCA at 4°C, for 30 min, then samples were filtered onto GFC filters (section 4.2.9), rinsed with 10 mls of 20% TCA at 4°C, followed by 95% ethanol at 4°C.

Table (ii) Comparison of DE81 filter and TCA precipitation assays

Assay procedure	C.p.m. per 100 μl of S_1 nuclease digest	
	dsDNA	ssDNA
DE81 filters	261 251 280	20
TCA precipitation	252 260 248	56

Results of the two assays were not significantly different, but indicated that the efficiency of the TCA precipitation assay may be as reasonable as the DE81 assay, and, possibly, more reproducible. Because of this and the comparative ease of the TCA precipitation procedure it was decided that the assay should be optimised.

(iii) Optimisation of the TCA precipitation assay of dsDNA

S_1 nuclease digestion was carried out as suggested in Appendix I and the TCA precipitation was varied to contain additional carrier in the 20% TCA precipitation (25 μg calf thymus DNA and 150 μg B.S.A./2 ml), to wash filters in either 5 or 20% TCA and to compare the efficiency of GFC and GFB filters.

Table (iii) Comparison of TCA precipitation conditions

Filter type	Carrier	TCA wash concentration	C.p.m. per 100 µl of S_1 nuclease digest dsDNA	ssDNA
GFC	✓	20%	1534(194)	54(9)
		5%	1322(57)	36(1)
	X	20%	2977(96)	71(10)
		5%	3033(128)	76(21)
GFB	✓	20%	1944(204)	50(9)
		5%	1897(180)	64(4)
	X	20%	2290(109)	52(1)
		5%	3042(70)	94(11)

Results are means of triplicate samples. Standard deviations are in parenthesis.

It was apparent that the high concentrations of carrier DNA and B.S.A. added to the TCA precipitation caused dsDNA counts to be either lost or quenched in scintillant. A comparison of filter types revealed that there was no advantage to be gained in using the thicker GFB filters, as regards maximal retention of precipitated DNA. It was also evident that both 20% and 5% TCA washes were equally as effective.

Discussion

These experiments indicated more suitable conditions for both the DE81 filter and TCA precipitation assays of dsDNA. No direct comparison was undertaken to determine which of the two procedures gave the least variation between replicates (see Maxwell *et al.*, 1978), although the TCA precipitation procedure gave reasonably low standard deviations.

APPENDIX IV

1. Kinetics of reassociations in solution

The rate of reassociation of denatured DNA in solution depends on the concentration of complementary DNA sequences: the rate-limiting step in duplex formation is a nucleation event which is followed by a rapid 'zippering' together of the two single strands (Chelm, 1982). Reassociations obey second order kinetics when (i) the concentrations of the two complementary strands are equal (which is true where only denatured, double stranded DNA (dsDNA) is able to reassociate) and (ii) all reassociating sequences are present in equal concentrations ('single component'). Therefore:

$$dC/dt = k(C)^2$$

where C is the concentration of single stranded DNA (ssDNA), k is the second order rate constant and t is time (Chelm, 1982).

The standard way of presenting reassociation kinetic data is the Cot curve, where the fraction of DNA remaining single stranded at time t, Ct/Co, is plotted against the initial DNA concentration times the reaction time, Cot. Since:

$$Ct/Co = \frac{1}{1 + kCot}$$

and

$$Cot\tfrac{1}{2} = \frac{1}{k}$$

it is possible to compare $Cot\frac{1}{2}$ values for different DNA samples with the $Cot\frac{1}{2}$ of a standard reassociation where the concentration of the reassociating sequence is known.

When S_1 nuclease digestion is used to assay the extent of reassociation, as was done here, the assay results do not accurately describe the second order reaction but they follow the pattern described by the equation:

$$Ct/Co = \frac{1}{(1 + kCot)^{-n}}$$

where n was found to be 0.45 (Young and Anderson, 1985). This is because single stranded tails reassociate at a rate two to four fold less rapidly than the wholly single stranded molecule. The hydroxyapatite chromatography technique for assaying reassociations, which is unable to distinguish these single stranded tails, follows the equation for a second order reaction. However, for both assays the $Cot\frac{1}{2}$ is still related proportionately to k.

2. Kinetics of hybridisations using filter bound RNA or DNA

The hybridisation of a double stranded probe to filter bound DNA or RNA is a function of two competing reactions: the reassociation of sequences in solution and hybridisation to filter bound sequences. The rate of disappearance of probe can be expressed.

$$-d[Cs]/dt = k_1[Cf][Cs] + k_2[Cs]^2$$

where Cf and Cs are the concentrations of complementary filter bound nucleic acid and probe, respectively, and k_1 and k_2 are the rate constants for filter hybridisation and solution hybridisation, respectively (Anderson and Young, 1985). Thus the initial rate of hybridisation is proportional to the concentrations of both probe and filter bound sequences. Where [Cf] is much greater than [Cs] the solution reassociation term can be ignored (e.g. plasmid blots). At low values of [Cf] the initial rate of hybridisation is proportional to [Cf], as predicted by the equation, however the rate does not increase linearly as [Cs] falls relative to [Cf], since probe diffusion, rather than the nucleation reaction, becomes the limiting process.

APPENDIX V

Percentage increase in grain fresh and dry weights and endosperm DNA content for C grains after grain removal

Grain age (d.p.a.)	Mean percentage increase		
	Fresh weight	Dry weight	Endosperm DNA content
9	8.7	-13.9	-
13	13.1	13.7	75.6
17	23.3	20.0	89.7
21	36.2	25.6	84.1
25	24.4	10.0	48.6
30	42.2	53.0	77.3
35	35.9	30.1	69.2
40	53.9	55.4	25.1
50	40.1	47.5	80.5
60	45.8	32.7	97.4
70	35.2	24.4	22.4
$\bar{x}$ from 9 to 70 d.p.a.	32.6±9.3	27.1±13.6	67.0±18.7

REPRINTED FROM

PLASTID DNA DURING GRAIN FILLING IN WHEAT

MANDY J. DOWSON DAY[a], MICHAEL W. BAYLISS[b] and JANET A. PRYKE[a,*]

[a]*Department of Biochemistry, University of Bath, Bath BA2 7AY and* [b]*ICI International Seeds Business, Jealott's Hill Research Station, Bracknell, Berks RG12 6EY (U.K.)*

(Received April 3rd, 1987)
(Revision received June 15th, 1987)
(Accepted July 27th, 1987)

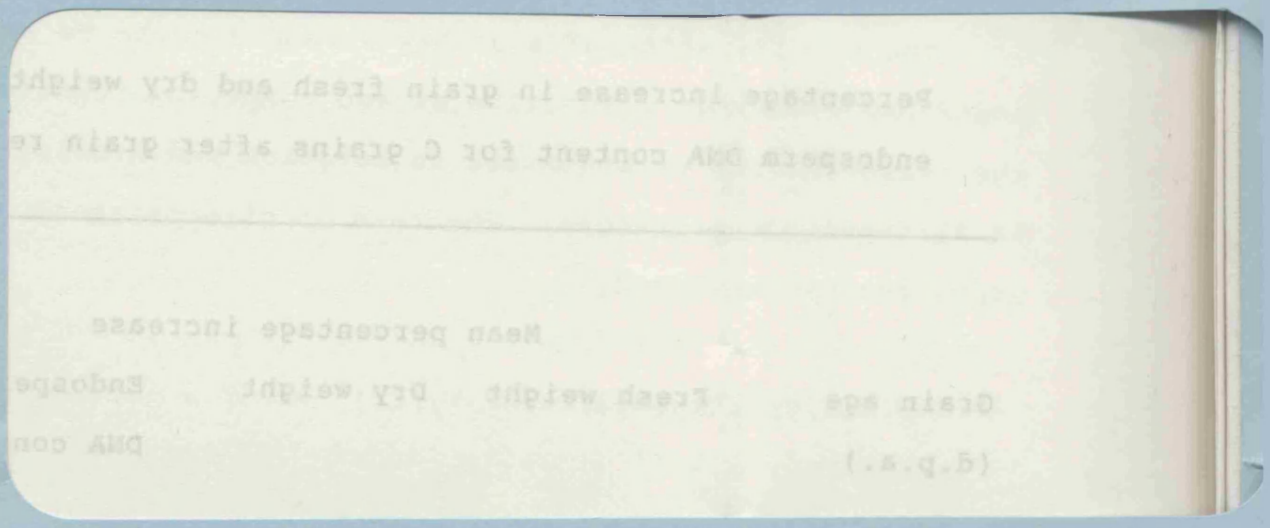
During grain filling in wheat (*Triticum aestivum* L.) there is a progressive increase in the number of amyloplasts in the endosperm, as well as in cell number, DNA content and nuclear ploidy as the grain increases in size. The plastid DNA content also rises initially, and then there is a levelling off in the amount, with the percentage plastid DNA finally making up approximately 0.9% of the total endosperm DNA.

PLANT SCIENCE

an international journal of experimental plant biology

ELSEVIER SCIENTIFIC PUBLISHERS IRELAND LTD

PLANT SCIENCE



Plant Science will publish, in the minimum time, papers recommended by its referees in all areas of experimental plant biology.

Papers describing experimental work with multicellular plants and plant-like microorganisms (protists, including algae and fungi and blue-green algae (cyanobacteria) are appropriate.

Submission of manuscripts in the broad areas of biochemistry (including enzymology), bioenergetics, cell biology, development, genetics, molecular biology, physiology and structure in relation to function as well as fundamental work in these areas related to agriculture and phytopathology is invited.

The following examples of areas covered by Plant Science Letters are not exhaustive; original work of high quality in any area of experimental plant biology, broadly construed, will be welcome.

Examples of areas covered by PLANT SCIENCE:

Cell and Tissue Culture, protoplasts, cell fusion
Cell organelles including development of plastids and mitochondria, cell walls
Cellular and Molecular Genetics
Enzymology and Intermediary Metabolism
Inorganic Metabolism including nitrate reduction, nitrogen fixation, sulfur metabolism, other macro and micro elements
Molecular biology of plant cells
Nucleic acids, proteins, and their metabolism
Nucleocytoplasmic interactions
Photosynthesis, respiration and carbon metabolism
Phytochemistry and secondary plant products
Pigments, including Biochemistry, Metabolism and Biosynthesis of Chlorophylls, Anthocyanins, etc.
Regulation of growth, development and differentiation
Ultrastructure and cellular fractionation
Viruses
Water relations, transport of solutes etc.

Plant Science, 53 (1987) 131–138
Elsevier Scientific Publishers Ireland Ltd.

PLASTID DNA DURING GRAIN FILLING IN WHEAT

MANDY J. DOWSON DAY[a], MICHAEL W. BAYLISS[b] and JANET A. PRYKE[a,*]

[a]*Department of Biochemistry, University of Bath, Bath BA2 7AY and* [b]*ICI International Seeds Business, Jealott's Hill Research Station, Bracknell, Berks RG12 6EY (U.K.)*

(Received April 3rd, 1987)
(Revision received June 15th, 1987)
(Accepted July 27th, 1987)

During grain filling in wheat (*Triticum aestivum* L.) there is a progressive increase in the number of amyloplasts in the endosperm, as well as in cell number, DNA content and nuclear ploidy as the grain increases in size. The plastid DNA content also rises initially, and then there is a levelling off in the amount, with the percentage plastid DNA finally making up approximately 0.9% of the total endosperm DNA.

Key words: wheat; *Triticum aestivum* L.; amyloplast; plastid DNA; endosperm

Introduction

The period of grain filling in wheat is a time of active biosynthesis of starch which is laid down as granules in the endosperm cells in a non-photosynthetic type of plastid, the amyloplast. The starch granules in amyloplasts from various plants take different forms, e.g. the close-packed small granules in root statoliths, and single starch granules in potato [1], while in wheat and other cereals the starch is stored in granules of two types: type A which are over 10 μm and type B less than 10 μm diameter [2,3]. There is a large increase in the number of both these types of granules during growth of the grain, and together they make up a large part (70–80%) of the final grain fresh weight [4,5]. There are accompanying increases in cell number, DNA, protein and other cell constituents [6].

We wished to investigate whether this increase in amyloplast number involved an accompanying increase in the amount of amyloplast DNA, which might imply the existence of some function for the plastid DNA in this category of non-photosynthetic plastids. Plastid DNA has been found in many types of non-photosynthetic plastid, including amyloplasts [7,8], though as yet it has no well defined coding role [9]. It has been thought that the amyloplasts in wheat arise by budding from previously existing amyloplasts, with subsequent separation of the daughter amyloplasts containing at least one already formed starch granule [10], however, recent observations indicate that complex connexions may continue to exist between amyloplasts containing A-type granules and what were previously thought to be separate amyloplasts containing B-type granules [11]. In chloroplasts, division of the organelles involves binary fission and partitioning of the DNA which is present in the plastid in several nucleoid structures [12], and such nucleoids have been observed in amyloplasts [7,8].

The techniques we used were analysis of various growth parameters in the developing grain: fresh and dry weight, DNA content, ploidy, starch granule number and type. The percentage plastid DNA was quantified using dot-blot hybridisation, and related to the development of the grain.

*To whom correspondence should be sent.

Abbreviations: SSC, 0.15 M NaCl, 0.015 M Na_3 citrate; PCA, perchloric acid; TCA, trichloroacetic acid

0618-9452/87/$03.50
Printed and Published in Ireland

Materials and methods

Growth of plants and preparation of endosperm tissue

Spring wheat (*Triticum aestivum* L. cv. Timmo) seeds were grown either in the greenhouse or in growth cabinets with 16 h light at 15°C and 8 h dark at 10°C. Tillers were removed leaving three stems per plant. On the day on which anthers were observed anthesis date was recorded, and the grains were sampled subsequently at intervals. Only grains from the first basal floret were used in these experiments. Grains were fixed in acetic acid/ethanol (1:3) stored at 4°C and used for nucleus counts, starch granule counts and ploidy measurements. Other grains were weighed and used for extraction of DNA and measurement of endosperm total DNA content.

Endosperms were prepared by peeling away all the layers of the pericarp, and stripping out the vascular and nucellar material in the crease. The embryo was discarded at all stages of grain development.

Analysis of nuclear DNA and cell number

Total DNA was measured using a modification of the Burton diphenylamine method, following perchloric acid (PCA) extraction of nucleic acids [13–15].

Nuclear ploidy was measured by using cytofluorimetry following Feulgen staining of the nuclei [16], taking measurements using a Leitz MPV 3 microscope fluorimeter, with a 200 W Hg lamp, 530–560 nm exciter filter and 580 nm suppression filter. Samples of 100 nuclei were measured for each batch of endosperm tissue, and compared with embryo and root nuclei as ploidy controls.

The cell numbers per endosperm were calculated using values of average nuclear C value to calculate the DNA content per nucleus, and the measured values of total DNA to give the number of nuclei and therefore the number of cells. It is assumed that the majority of endosperm cells are mononucleate.

Starch granule number and size distribution

Starch granules were prepared from tissue that had been first fixed in acetic acid/ethanol (1:3), rehydrated through an alcohol series, digested at 37 °C for 15 h in citrate–phosphate buffer (pH 5), containing 1% cellulase and 1% Macerozyme (both from Onozuka Chemicals), then centrifuged at 13 000 for 3 min, and the starch granule pellet resuspended [5]. This was repeated 3 times. The granules were measured and counted using a Coulter counter (Model TAII) previously calibrated with particles of known diameter [16]. Numbers of A-type granules were calculated from the proportion of the granule population having a size greater than 10 μm diameter.

DNA extraction

The endosperms were homogenised in 0.1 M Tris–HCl, 5 mM EDTA, 1% Na sarcosinate, 0.1 mg ml^{-1} ethidium bromide and 1 mg ml^{-1} protease (pH 8.0) at 4°C, and then extracted for 15 min three times with buffered phenol, the interface material being included in the re-extractions. The aqueous layer was then extracted twice with chloroform/isoamyl alcohol (24:1). DNA was separated from residual protein and RNA using CsCl ethidium bromide density gradient centrifugation, which gave a clear RNA pellet and DNA band. The DNA was removed from the tube with a syringe. Nuclear DNA to use as a background control for the dot-blot hybridisations was extracted from the tissue as described by Luthe and Quatrano [17]. The DNA was further purified by treatment with restriction enzyme Hpa II which does not cut methylated nuclear DNA, while it does restrict plastid DNA [18].

Dot-blot hybridisations

Probe DNA was either chloroplast fragment P6 or B2, a gift from T.A. Dyer [19]. These were nick-translated using either [^{35}S]dCTP or [^{32}P]dATP, and the labelled DNA separated from nucleotides using chromatography on a Sephadex G50 column.

DNA to be probed was added to the filters

to give radioactivity hybridising in the range 100—3000 counts min^{-1}/spot. Replicate samples were applied in serial dilutions on each filter, using a Schleicher and Schuell filtration manifold, after denaturation in 2 M NaOH, and neutralisation with acetic acid. The filters used were Pall Biodyne A. The filters were washed with 15 × SSC and dried in a vacuum oven at 80°C. Probe DNA was added to the filters following prehybridisation for 4—6 h in the presence of 0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin and 500 $\mu g\ ml^{-1}$ salmon testis DNA in 0.9 M NaCl, 0.005 M EDTA, 0.05 M sodium phosphate (pH 8.3) at 65°C, and incubation was carried out in the same buffer for 18—24 h. Each filter was washed three times in 2 × SSC and 0.1% SDS, with shaking, followed by a 30-min wash at 50°C in 0.1 × SSC and 0.1% SDS. It was then dried and subjected to autoradiography. Using the autoradiograph as a template, the areas containing plastid DNA were cut from the hybridisation filter and the amounts of bound label estimated by scintillation counting. The counts measured were found to be directly proportional to the amount of DNA on the filter.

The percentage plastid DNA made of the total was calculated by comparison with hybridisation to a dilution series of probe DNA, and the values corrected for the proportion of the plastid DNA made up by the probe.

Results

During grain filling there is an increase in grain size, as described by the fresh and dry weight curves in Fig. 1, up to 25 days after anthesis. This is the time period over which we made our observations on other growth parameters, relating them to grain fresh weight and therefore developmental stage. At later stages of grain development the weight stabilises and the grains start to dehydrate [20].

The method adopted for extraction and measurement of total DNA in the endosperm involved several extractions of the insoluble

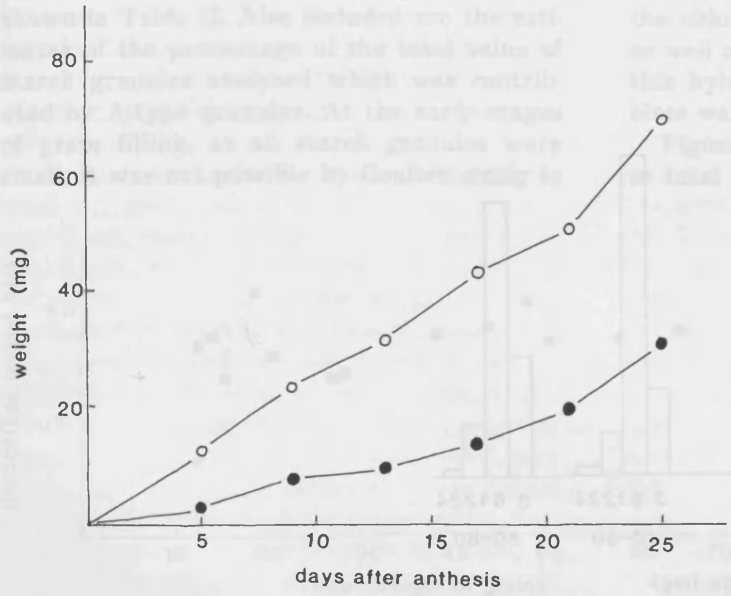


Fig. 1. The growth of the basal grains as used in the dot-blot hybridisation experiments as measured by fresh (○) and dry (●) weight over the period 0—26 days after anthesis.

Table I. Calculation of cell number per endosperm during grain development, using the values of average ploidy, and the total DNA content of the endosperm. S.D. of estimations are in brackets.

	Fresh weight range of grains (mg)							
	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80
Total DNA content of endosperm (μg)	1.2	3.33 (2.43)	11.93 (0.89)	13.34 (1.39)	17.20 (1.73)	19.79 (0.57)	23.90 (1.80)	25.27 (3.00)
Average nuclear ploidy (C)	3.27	3.90	4.50	4.95	5.25	5.25	5.25	5.25
No. of cells/ endosperm ($\times 10^5$)	0.212	0.494	1.53	1.56	1.89	2.18	2.63	2.78

cellular material and this was found to be necessary for maximal DNA yields. Two methods were used to measure the cell number in the developing endosperm: using the total DNA, together with cytophotometric measurements of nuclear ploidy to calculate the number of nuclei; and direct microscopic counting of extracted Feulgen-stained nuclei. The results of the two methods were found to be comparable in younger grains (up to 20 mg fresh wt.) but the results from the DNA extractions gave higher values at later stages of grain development, when the high volume of starch grains obscured the nuclei in the direct counting technique. The DNA extraction method was adopted for our analysis. Table I shows the increase in total DNA in the endosperm as related to the fresh weight of the grains.

Figure 2 shows the C value distributions of the endosperm nuclei at different stages of the development of the grain. The triploid endosperm initially shows the majority of nuclei in the 3C and 6C categories with an average C value of 3.90C. As grain growth proceeds, increasing numbers of nuclei appear in the 6C, 12C and eventually 24C categories leading to a final average C value of 5.25C.

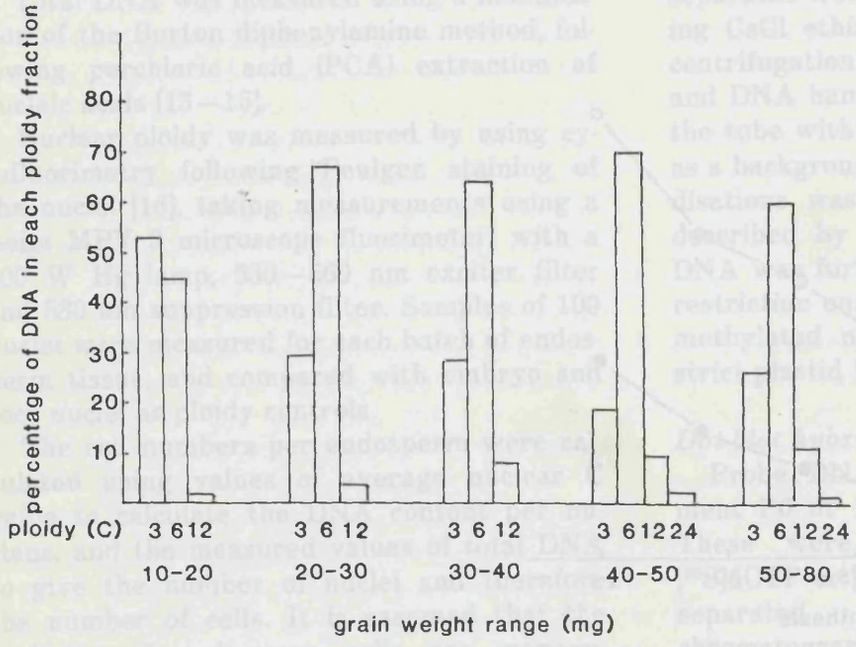


Fig. 1. C-values of endosperm nuclei at successive stages of grain development. The DNA content of samples of 100 nuclei for each 10 mg size range were measured using cytofluorimetry following Feulgen staining.

Table II. Starch granule numbers during endosperm development. Grains were measured and counted in a Coulter counter. S.D. of 4 estimations; ND not determined.

	Fresh wt. range of grains (mg)							
	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80
Total no. of starch granules ($\times 10^6$)	0.0354 (0.03)	0.421 (0.17)	6.98 (1.55)	10.85 (2.20)	18.46 (1.82)	110.6 (28.3)	146.2 (36.5)	193.6 (46.2)
% of starch granule no. which are A-type	ND	2.33 (0.68)	18.78 (4.20)	27.43 (3.08)	25.13 (4.57)	5.50 (1.95)	5.25 (1.20)	4.38 (1.06)
% of starch volume contributed by A-type granule	ND	66.5 (2.77)	73.2 (11.10)	90.0 (1.56)	92.1 (1.37)	79.2 (7.7)	78.6 (5.09)	72.6 (7.36)

Average nuclear DNA content for the endosperm was calculated from the average C values using a value of 17.3 pg for the 1C quantity of wheat nuclear DNA [21]. Table I describes the relationship between the average C value of the grains and their fresh weight. The DNA content per endosperm divided by the average DNA-content per nucleus was used as an estimate of cell number per endosperm for the different categories of grain, and these values are also presented in Table I.

The numbers of starch granules and the proportion of A-type starch granules are shown in Table II. Also included are the estimates of the percentage of the total value of starch granules analysed which was contributed by A-type granules. At the early stages of grain filling, as all starch granules were small, it was not possible by Coulter sizing to distinguish those granules which eventually grew to A-type size. Subsequently, in grains exceeding a fresh weight of 50 mg, an approximately constant proportion of granules (both by number and volume) could be classified as A-type.

A preliminary experiment to investigate the presence of plastid DNA in endosperm was carried out in which a Southern blot hybridisation was carried out, on total DNA from both leaf and endosperm, restricted with Pst 1, and probed with cloned P6 fragment. There was clear hybridisation to a single 8.4 kilobase (kb) band, indicating the presence of the chloroplast DNA fragment in endosperm as well as in leaf plastids. In order to quantify this hybridisation a method using DNA dot blots was used.

Figure 3 gives the percentage plastid DNA in total endosperm plotted against the fresh

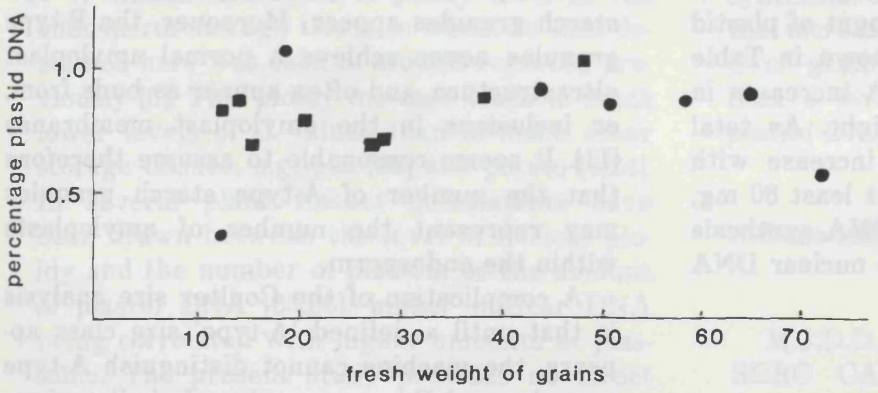


Fig. 3. Percentage plastid DNA as detected by dot blots of probe plastid DNA to total endosperm DNA. ■, ●, samples from two different time courses.

Table III. Estimation of the average amount of plastid DNA per cell and per amyloplast.

	Fresh wt. range of grains (mg)							
	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–
Amount of plastid DNA (ng)	–	25	107	121	156	180	217	225
Average plastid DNA per cell								
(a) g × 10^{-13}	–	4.98	7.01	7.78	8.28	8.27	8.27	8.16
(b) no. plastid genomes	–	2960	4170	4630	4930	4920	4920	4860

weight of grains. This was calculated from the dot-blot experiments, by comparing the radioactivity hybridising to a dilution series of total DNA from endosperms at different stages, with the radioactivity hybridising to a dilution series of unlabelled probe.

We carried out parallel hybridisations where the probes were both hybridised to endosperm and leaf nuclear DNA, prepared as in Materials and methods. There was a low level hybridisation with both probes (an average of 0.38% with P6, and 0.34% with B2) and this was subtracted from the values of percentage plastid DNA, to give the values in Fig. 3. Figures for each point are the mean values of percentage plastid DNA obtained from 4 replicate hybridisations to both the B2 and P6 wheat chloroplast DNA probes. Using this technique and hybridisation to wheat leaf DNA we consistently obtained values of 11–12% plastid DNA.

In grains weighing greater than 20 mg the proportion of plastid DNA remains approximately constant at 0.9% of total endosperm DNA. Using the values of total DNA per endosperm the absolute amount of plastid DNA was estimated. This is shown in Table III. The amount of plastid DNA increases in the same pattern as fresh weight. As total endosperm DNA continues to increase with endosperm fresh weight up to at least 80 mg, the implication is that plastid DNA synthesis is occurring at a similar rate to nuclear DNA synthesis during grain filling.

Discussion

The basal grains studied here show a pattern of development during grain filling s lar to that previously described in wheat other cereals, [25,26]. Although the bio thesis of starch takes place inside amyloplast, all the genes so far studied v respect to starch biosynthesis are nucl coded, and the mRNAs are therefore tr lated on cytoplasmic ribosomes and the res ing polypeptides transported into the pla [16]. As far as the biosynthesis of starcl concerned there are no known roles for p ucts encoded by the plastid genome of amyloplast.

In the current study, the quantity of p tid DNA increased early during grain fill and later reached a plateau while the gr was still increasing in weight. It is not kn whether the plastid DNA is divided betw the different forms of amyloplast (as cha terised by large and small starch grains), any estimation of the plastid DNA per am plast can only be an approximation. Ear studies (Refs. 3,5,22; Bayliss, unpublished servation) have indicated that there is a de in endosperm development before the B-t starch granules appear. Moreover, the B-t granules never achieve a normal amylopl ultrastructure, and often appear as buds fr or inclusions in the amyloplast membra [11]. It seems reasonable to assume theref that the number of A-type starch granu may represent the number of amylopla within the endosperm.

A complication of the Coulter size analy is that until a defined 'A-type' size class pears, the machine cannot distinguish A-t granules and B-type granules of similar siz Thus it can neither reliably pin-point which

type granules first appear, nor give accurate estimates of A-type numbers during early grain growth [5]. As A-type amyloplasts are initiated very early during grain filling [22] an estimate of the number of these amyloplasts during grain filling is the number calculated for the oldest grains (i.e. 8.48×10^6/endosperm). However, as this number is larger than the total number of starch granules observed at early stages, there is then obviously still some plastid differentiation and/or division occurring involving precursor plastids, and we cannot estimate the copy number of plastid genome per A-type plastid early in grain filling. Using the values for later stages of development the amount of plastid DNA per A-type amyloplast rises from 1.41×10^{-14} g in grains of 30–40 mg fresh wt. to 2.65×10^{-14} g in grains of 70–80 mg fresh wt. Using a plastid genome weight of 1.68×10^{-16} g [23], this may be 84 rising to 158 genome copies per plastid.

It should also be noted that during the later stages of grain filling, when the number of B-type granules increases dramatically (approximately 10-fold between grain weights of 40 and 70 mg, as shown in Table II) there is only a relatively small increase in plastid DNA content in the endosperm (from 155 to 225 ng as shown in Table III). Given the assumptions above in interpretation of granule size categories, it seems probable that the formation of B-type starch granules occurs without concurrent plastid DNA replication.

A similar elevation of ploidy level in the endosperm storage tissue in wheat to that described here was seen in studies reported previously [6]. This ploidy increase leads to much lower levels of C values than in some other storage tissues, e.g. pea [23] and potato [6,24]. In several plant tissues correlations have been drawn between the level of nuclear ploidy and the number of plastids or the amount of plastid DNA [25,26], higher nuclear DNA being correlated with higher amounts of plastome. The present study provides no direct evidence for such an interrelationship in wheat endosperm. However, as the measurements made represent an average across the various cell types within the endosperm, such a relationship cannot be excluded.

Comparison with studies in the plastid genome in the amyloplasts of potato tubers shows that the estimated number of plastome copies per cell in wheat endosperm (2960–4930) is as that in potato at a similar level to tuber amyloplasts (7800–18 000 copies/cell) [6,24], and the average plastome number per A-type amyloplast at the later stages of grain filling, i.e. copies of plastid DNA, 158 is comparable to that calculated for potato (195 copies/amyloplast [6]). These results for the plastid DNA content in wheat endosperm can be compared to the patterns of plastid replication seen during leaf development in wheat [27]. During leaf development, the plastid genome copy number per plastid decreases from approximately 1000 in plastids within basal meristematic cells to approximately 300 in mature chloroplasts. Interestingly, a recent report [28] in another wheat variety, has estimated that mature chloroplasts contain approximately 150 genome copies, so that final values observed here in endosperm may represent a functionally effective copy number for mature organelles.

In conclusion, our analysis of plastid DNA during grain filling in wheat has shown that there is initially a large increase in the quantity of plastid DNA in the developing endosperm. This synthesis, however, appears to be more closely coordinated with nuclear DNA synthesis, or the increase in cell number, than the increase in numbers of starch granules. Over grain filling it leads to an initial rise, then a levelling of the average number of plastid DNA molecules per cell.

Acknowledgements

M.J.D.D. acknowledges the receipt of a SERC CASE Studentship. We thank W. Schuch, C.M. Bowman and K. Edwards for helpful discussions on the work.

References

1 J.T.O. Kirk and R.A.E. Tilney Bassett, The Plastids, Elsevier, Amsterdam, 1978.
2 A.D. Evers, Starch, 23 (1971) 157–162.
3 L.H. May and M.S. Buttrose, Aust. J. Biol. Sci., 12 (1959) 146–159.
4 C.F. Jenner, Storage of starch, in: F.A. Loewes and W. Tanner (Eds.), Encyclopaedia of Plant Physiol. N.S. 13A. Plant Carbohydrates I, Springer, pp 700–737.
5 A.J.S. Chojecki, M.D. Gale and M.W. Bayliss, Ann. Bot., 58 (1986) 819–832.
6 A.J.S. Chojecki, M.W. Bayliss and M.D. Gale, Ann. Bot., 58 (1986) 809–818.
7 N. Steele Scott, M.J. Tymms and J.V. Possingham, Planta, 161 (1984) 12–19.
8 D. Macherel, H. Kobayashi, T. Akazawa, S. Kawano and T. Kuroiwa, Biochem. Biophys. Res. Commun., 133 (1985) 140–146.
9 D. Macherel, H. Kobayashi, E. Valle and T. Akazawa, FEBS Lett., 201 (1986) 315–320.
10 M.S. Buttrose, Aust. J. Biol. Sci., 16 (1963) 305–317.
11 M.L. Parker, J. Cereal Sci., 3 (1985) 271–278.
12 S.A. Boffey, in: J. Bryant and D. Francis (Eds.), The Cell Division Cycle in Plants, 1985, pp. 233–246.
13 R.M. Smillie and G. Krotkov, Can. J. Bot., 38 (1960) 31–49.
14 K.W. Giles and A. Myers, Nature, 206 (1965) 93.
15 K. Burton, Biochem. J., 62 (1956) 315–323.
16 N. Bohm and E. Sprenger, Histochemie, 16 (1968) 100–118.
17 D.S. Luthe and R.S. Quatrano, Plant Physiol., 65 (1980) 305–308.
18 Y. Gruenbaum, T. Naveh-Many, H. Cedar and A. Razin, Nature 292 (1981) 860–862.
19 C.J. Howe, C.M. Bowman, T.A. Dyer and J.C. Gray, Mol. Gen. Genet., 190 (1983) 51–55.
20 P.A. Brocklehurst, Nature, 266 (1977) 348–349.
21 M.D. Bennett and J.B. Smith, Phil. Trans. Roy. Soc. B., 274 (1976) 227–274.
22 L.G. Briarty, C.E. Hughes and A.D. Evers, Ann. Bot., 44 (1979) 641–658.
23 J.R. Bedbrook and R. Kolodner, Annu. Rev. Plant Physiol., 30 (1979) 593–620.
24 J.C. Conover and J.A. Pryke, J. Exp. Bot., 38 (1987) 1219–1227.
25 A. Scharpe and R. Van Parijs, J. Exp. Bot., 24 (1973) 216–222.
26 T.M. Butterfass, Patterns of chloroplast reproduction, Cell Biology Monographs. VI, Springer, 1979.
27 A.J. Jellings and R.M. Leech, New Phytol., 96 (1984) 371–382.
28 C.M. Bowman, Planta, 167 (1986) 264–274.

NOTE TO AUTHORS

Manuscripts should be sent to one of the following addresses:
Prof. J.A. Schiff-Institute for Photobiology, Brandeis University, WALTHAM, MA 02154 (U.S.A.)
Prof. J.H. Weil-Institut de Biologie Moléculaire des Plantes, 12 Rue du Général Zimmer, 67000 STRASBOURG (France)

Manuscripts in English, should be submitted in *triplicate* in double-spaced typing. Papers should normally be subdivided into sections: summary, introduction, methods, results, discussion. Author's full names, academic or professional affiliations and addresses should be included on the first page. Indication of name and address for proofs should be given. Authors should indicate when submitting papers in which of the following sections they wish their paper to be listed: Physiology and Biochemistry; Genetics and Molecular Biology; Cell and Tissue Studies in vitro; or General. Authors should consult papers in a recent issue for details of style that are not printed below. Authors are responsible for submitting well-written manuscripts in proper style that have been carefully read and corrected to eliminate errors. Authors who are not fluent in English should have their manuscripts read by English-speaking colleagues to insure that the submitted paper is free from grammatical errors. Failure to meet these standards may cause serious delay in the handling of manuscripts; manuscripts with serious deficiencies will be returned to the authors for rewriting before they are considered further.

Submission of a paper to *Plant Science* is understood to imply that it is not being considered for publication elsewhere and that the author's permission to publish his/her article(s) in this journal implies the exclusive authorisation of the publisher to deal with all issues concerning the copyright therein. Submission of multi-authored manuscripts to the journal implies the consent of *each* of the authors. The publisher will assume that the senior or corresponding author has specifically obtained the approval of all other co-authors to submit the manuscript to this journal.

Summary The article should start with a summary of approx. 100–200 words.

Key words: a maximum of six key words (or short phrases) should be included with the submitted manuscript. These will be used in the compilation of the subject index and for any subsequent retrieval.

Figures should be submitted in triplicate. Line drawings (including graphs) should be drawn in black ink on white paper. Alternatively sharp photoprints may be provided. The lettering should be large enough to permit photographic reduction. Half-tone illustrations may be included. They should be submitted as clear black-and-white prints on glossy paper. Minimum size of figures: 4 X 5" or 10 X 12 cm. All legends should be typed on a separate sheet.

Tables should bear a short descriptive title and should also be typed on separate sheets.

References should be numbered singly using numerals in square brackets and listed in numerical sequence on a separate sheet. References should be cited in full in the reference list, including the title and the first and last page numbers.
In the reference list, periodicals [1], books [2], and multi-author books [3] should accord with the following examples:

1 J.F. Briat, M. Dron and R. Mache, Is transcription of higher plant chloroplast ribosomal operons regulated by premature termination? FEBS Lett., 163 (1983) 1–5.
2 T. Bengochea and J.H. Dodds, Plant Protoplasts, Chapman & Hall, London, New York, 1986, 1–90.
3 H.J. Bohnert, E.J. Crouse and J.M. Schmitt, Organization and expression of plastid genomes, in: B. Parthier and D. Boulter (Eds.), Encyclopedia of Plant Physiology, New Series, Vol. 14B, Springer Verlag, 1982, pp. 475–530.

Abbreviations for the titles of journals should follow the system used by *International Series Catalogue,* 1978 edition and supplements.

Page charge There will be no page charge.

Reprints Upon acceptance of a paper for publication the author will receive an order form on which he can order reprints above the 50 free reprints of each article allowed.

Proofs Authors will receive proofs which they are requested to please return within 48 hours of receipt.

Advertisements For advertising rates apply to the publishers.

ORDER FORM

Send to your usual supplier or: **ELSEVIER SCIENTIFIC PUBLISHERS IRELAND LTD.**, P.O. Box 85,Limerick, Ireland.

For further information and/or free specimen copy write to, in the U.S.A. and Canada: Journal Information Center, **Elsevier Science Publishing Co. Inc.**, P.O. Box 1663, Grand Central Station, New York, NY 10163, U.S.A. In the rest of the world: **Elsevier Scientific Publishers Ireland Ltd.**, P.O. Box 85, Limerick, Ireland.

Journals are automatically sent by air to the U.S.A. and Canada at no extra cost and to Japan with a small additional charge. Airmail rates for other countries are available on request.

PLANT SCIENCE

Volumes 53–57 (5 vols. in 15 issues)

Orders from individuals must be sent direct and accompanied by a remittance.

☐ Please enter a subscription for 1988 at US$760.00 including postage and handling.
☐ Please send me a proforma invoice

☐ I enclose: ☐ **My Personal Cheque** ☐ **Bank Draft**

☐ Please bill my credit card

☐ **Mastercard** ☐ **Eurocard** ☐ **Visa** ☐ **American Express** ☐ **Access**

Card No. . . . . .Valid Until. . . . . .

Name . . . . . .

Address. . . . . .

. . . . . .

. . . . . Postal/Zip Code. . . . . .

Date . . . . . .Signature. . . . . .

US$ price is definitive

RP

RECOMMENDATION TO LIBRARIAN

I would like a sample copy of: **PLANT SCIENCE**
Please send to the Librarian at my institute with my recommendation to subscribe.

My Name. . . . . .

My Department. . . . . .

Name of Library . . . . . .

Address. . . . . .

. . . . . .

. . . . . .Postal/Zip Code . . . . . .

Date . . . . . .Signature. . . . .

RP